out of 21, three very homologous regions can be defined (Fig. 5b). From the deduced amino acid sequence we located the first such region from aa residues 266 to 350 (85 aa), the second from aa residues 403 to 527 (125 aa), and the third from aa residues 641 to 702 (62 aa). To investigate whether there is a biological significance to this high amino acid homology we computed the K_a values (number of substitutions per nonsynonymous site) and the K_s values (number of substitutions per synonymous site) for different regions of the genes compared (Li et al., 1985). Comparisons between K_a and K_s values give indications of the degree of functional constraints placed on the different regions analysed. Table I shows the nucleotide sequence and the amino acid sequence homology, and the K_a and K_s values for the comparison of six different regions between the maize and potato sucrose synthase genes. The location of these regions is shown in Fig. 4. The six different regions, numbered from 1 to 6, show about the same nucleotide sequence homology (73%) when maize and potato are compared (except for region 3: 76%, and region 6: 68%). At the amino acid level the situation is quite different, indeed regions 1, 3 and 5 are about 90% homologous while regions 2, 4 and 6 are homologous at only 70% or less. Regions 1, 3 and 5 correspond to the three well-conserved regions described earlier. Meaningful estimates of K_s values can be obtained only for regions 2 and 3 because of the high degree of mutation occurring at the four-fold degenerate site for the other regions (transition as well as transversion at a four-fold degenerate site never causes an amino acid change). The ratio K_s/K_a is 34 for region 3 and 4 for region 2. The ratio K_s/K_a for the very slow evolving histone genes analysed by this method varies from 227 to 48 and the average K_s/K_a ratio computed for 37 mammalian genes is 5.3 (Li et al., 1985). The values K_s/K_a found for regions 2 and 3 show that these two protein regions evolved at a different rate, region 2 evolved at a rate comparable to the average of mammalian genes while region 3 evolved more slowly although the rate of divergence of the two gene regions reflected by the K_s values is comparable for these two regions. The slow evolution of the part of the protein encoded by region 3 is most probably due to a high constraint on the amino acid sequence, and suggests that this part of the protein is essential for the protein function. Even if the K_s values are not available for the four other regions the K_a values for regions 5 on the one hand, 4 and 6 on the other hand suggest that the parts of the protein encoded by region 5 evolved at a comparable rate to the one encoded by region 3 and that the parts of the protein encoded by regions 4 and 6 evolved at a rate comparable to the one encoded by region 2. The average K_a value for regions 3 and 5

TABLE I

Nucleotide and amino acid sequence similarity between maize and potato sucrose synthase gene

Region 1	Region 2	Region 3	Region 4	Region 5	Region 6
Nucleotide sequence homology (%)[a]					
72.9	72.4	76.5	72.8	73.7	67.8
Amino acid sequence homology (%)[b]					
88.2	65.4	90.0	70.7	90.0	65.5
Number of substitutions per synonymous (K_s) and non synonymous (K_a) site[c]					
K_s NA	1.12 (0.037)	2.51 (0.096)	NA	NA	NA
K_a 0.12 (0.03)	0.22 (0.05)	0.07 (0.02)	0.20 (0.03)	0.07 (0.02)	0.24 (0.05)

[a] Number of matching bp between the two nucleotide sequences in this region / number of bp of this region.

[b] Number of matching amino acids between the 2 aa sequence in this region / number of amino acids of this region.

[c] K_s and K_a values were calculated using the computing program provided by and described in Li et al. (1985). Values in parentheses are standard errors. NA, not applicable (no meaningful estimate could be obtained by this method).

is 0.069, i.e., more than three times lower than the average K_a value for regions 2, 4, and 6. On the other hand the average percentage of mutation per synonymous site is higher for regions 3 and 5 than for regions 2, 4, and 6. For region 1 the data do not allow clear conclusions to be drawn. These results suggest that the parts of the protein encoded by regions 3 and 5 are highly conserved due to strong constraints at the amino acid sequence level and are likely to be important for enzyme function.

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Single nucleotide polymorphism (SNP) genotyping as basis for developing a PCR-based marker highly diagnostic for potato varieties with high resistance to *Globodera pallida* pathotype Pa2/3

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Abstract *Globodera pallida* is a parasitic root cyst nematode of potato, which causes reduction of crop yield and quality in infested fields. Field populations of *G. pallida* containing mixtures of pathotypes Pa2 and Pa3 (Pa2/3) are currently most relevant for potato cultivation in middle Europe. Genes for resistance to *G. pallida* have been introgressed into the cultivated potato gene pool from the wild, tuber bearing *Solanum* species *S. spegazzinii* and *S. vernei*. Selection of resistant genotypes in breeding programs is hampered by the fact that the phenotypic evaluation of resistance to *G. pallida* is time consuming, costly and often ambiguous. DNA-based markers diagnostic for resistance to *G. pallida* would facilitate the development of resistant varieties. A tetraploid F_1 hybrid family SR-Gpa segregating for quantitative resistance to *G. pallida* was developed and evaluated for resistance to *G. pallida* population 'Chavornay'. Two subpopulations of 30 highly resistant and 30 susceptible individuals were selected and genotyped for 96 single nucleotide polymorphism (SNP) markers tagging 12 genomic regions on 10 potato chromosomes. Seven SNPs were found significantly linked to the nematode resistance, which were all located within a resistance 'hotspot' on potato chromosome V. A haplotype model for these seven SNPs was deduced from the SNP patterns observed in the SR-Gpa family. A PCR assay 'HC' was developed, which specifically detected the SNP haplotype *c* that was linked with high levels of nematode resistance. The HC marker was only found in accessions of *S. vernei*. Screening with the HC marker 34 potato varieties resistant to *G. pallida* pathotypes Pa2 and/or Pa3 and 22 susceptible varieties demonstrated that the HC marker was highly diagnostic for presence of high levels of resistance to *G. pallida* pathotype Pa2/Pa3.

Keywords Potato · *Globodera pallida* · Root cyst nematodes · Single nucleotide polymorphism (SNP) · Diagnostic marker · Haplotype

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Introduction

Two closely related root cyst nematode species, *Globodera rostochiensis* and *Globodera pallida*, are the most damaging parasitic nematodes in potato cultivation (Evans and Trudgill 1992). Control by nematicides and crop rotation is difficult, due to the high toxicity of nematicides to the environment and the long survival rate of the cysts in the soil. Therefore, high levels of genetic resistance to nematodes are an important character in breeding new potato varieties. This breeding goal has been successfully achieved for resistance to *Globodera rostochiensis* by introgression of major genes for resistance from *S. tuberosum* ssp *andigena*, *S. spegazzinii* and *S. vernei* (Ross 1986; Phillips 1994). The wide spread cultivation of varieties with resistance to *G. rostochiensis* favored however the multiplication of pathotypes of *G. pallida*, which gradually overcome these resistance genes. Resistance to *G. pallida* has been identified in the same *Solanum* species as resistance to *G. rostochiensis*, and accessions of *S. vernei* have mainly been used in variety breeding in the Netherlands and Germany (Ross 1986). Resistance to nematodes is assessed by the inoculation of test plants with a defined number of cysts or by planting in nematode infested soil. The cysts newly formed after 4–6 weeks post inoculation are collected and counted. Resistance to *G. pallida* appears quantitative and its phenotypic assessment is complicated by the fact that it depends not only from the genotype of the potato plant tested but also from the nematode population used for inoculation. Three pathotypes Pa1, Pa2 and Pa3 of *G. pallida* have been distinguished based on differentials (Kort et al. 1977). *G. pallida* field populations are however not uniform with respect to pathotype composition. Populations containing mixtures of pathotypes Pa2 and Pa3 (Pa2/3) are currently the most relevant for potato cultivation in middle Europe. The phenotypic evaluation of resistance to *G. pallida* is time consuming, costly and often ambiguous. The availability of DNA-based markers, which are easy to score, cost effective and diagnostic for resistance to *G. pallida* in wide germplasm pools, would reduce the need for resistance testing during variety development.

A prerequisite for developing such markers is the genetic dissection of the factors conferring resistance to *G. pallida*. A number of QTL (quantitative trait locus) mapping experiments for resistance to *G. pallida* have been performed in different, mostly diploid genetic backgrounds (Kreike et al. 1994; Bradshaw et al. 1998; Rouppe van der Voort et al. 1998, 2000; Bryan et al. 2002, 2004; Caromel et al. 2003, 2005). The major gene *Gpa2* for resistance to *G. pallida* pathotype Pa2 on chromosome XII has been cloned and shown to be a member of the NB (nucleotide binding) – LRR (leucine-rich-repeat) gene family, to which most known plant genes for pathogen resistance belong (van der Vossen et al. 2000). All QTL mapping studies for *G. pallida* resistance revealed one QRL (quantitative resistance locus) of major effect accompanied by few additional QRL of minor effect. The most prominent and reproducible QRL is located on potato chromosome V in a genomic region flanked by RFLP markers GP179 and GP21. A number of qualitative and quantitative resistance factors to various pathogens maps to this region as well as NB-LRR type genes (reviewed in Gebhardt and Valkonen 2001, https://www.gabi.rzpd.de/projects/Pomamo/). Other QRL with large effects were detected on chromosomes IV, IX and XI. RFLP (restriction fragment length polymorphism), AFLP (amplified fragment length polymorphism) SSR (simple sequence repeat) and other PCR-based markers linked to QTL for resistance to *G. pallida* are available in abundance from these mapping experiments. However, a diagnostic value of a DNA marker beyond a particular QTL mapping population has been demonstrated only for the marker SPUD1636 linked to the major QRL on chromosome V (Bryan et al. 2002). In this case, an allele specific, 226 bp amplicon was found only in accessions of *S. vernei* and in some highly resistant breeding lines that have *S. vernei* as source of resistance to *G. pallida* in their pedigree (Bryan et al. 2002).

Single nucleotide polymorphism (SNP) markers have been identified in potato, which tag most of the genomic regions known to harbor genes for qualitative and/or quantitative resistance to various pathogens, among others resistance to *G. pallida*. Most of these SNP markers are derived

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from end sequences of potato BAC (bacterial artificial chromosome) insertions, which contain one or more NB-LRR type genes (Rickert et al. 2003). Due to the high degree of intraspecific DNA polymorphism in potato, comparative sequencing of specific amplicons generated from genomic DNA of different genotypes allows the simultaneous scoring of several SNP markers in a sequence of 200–500 bp. In addition, the sequence trace files can be evaluated at heterozygous SNP loci for the three possible allele dosages in tetraploid genotypes (3:1, 2:2 and 1:3) (Rickert et al. 2003). Taking advantage on one hand of the current knowledge of 'resistance hot spots' in the potato genome and on the other hand of SNP markers linked to them, we selectively genotyped resistance-linked SNP markers in two subpopulations of highly resistant and susceptible individuals of a tetraploid F_1 family, which segregated for quantitative resistance to *G. pallida*. We identified SNP markers linked to a major resistance locus and used the SNP information to develop a haplotype specific PCR assay, which we show to be highly diagnostic for presence of high Pa2/3 resistance to *G. pallida* that has been introgressed in modern potato varieties from *Solanum vernei*.

Materials and methods

Plant material

The F_1 family Gpa-SR was generated by crossing the tetraploid breeding clones SR6 and SR5. SR6 was resistant to *Globodera pallida* pathotypes Pa2 and Pa3 and SR5 was susceptible. Of 250 seedlings grown in the greenhouse and assessed for general plant vigor, 20 plants were discarded. The remaining 230 genotypes were tested for resistance to *G. pallida* (one replication per genotype). Based on the first resistance test, 200 genotypes were retained and propagated the following year in the field at Windeby, Germany, under the same phytosanitary conditions as used for seed potatoes. The 200 genotypes were tested a second time for resistance to *G. pallida* (two replications per genotype). Based on the results of the two rounds of testing, 30 highly resistant and 30 highly susceptible genotypes were selected, which were propagated a third year and tested a third time for resistance to *G. pallida* (four replications per genotype). Based on a total of seven resistance tests, the average cyst counts were between zero and one for the highly resistant genotypes, and 22 cysts or more in the susceptible genotypes.

Thirty four varieties with resistance to *G. pallida* pathotypes Pa2 and/or Pa3 (Table 1) were obtained from Saka-Ragis Pflanzenzucht, Böhm-Nordkartoffel Agrarproduktion (BNA), Bavaria Saat (by courtesy of A. von Zwehl), HZPC Holland B.V., Averis Seeds B.V.and B.F. Leestemaker & A. Smid (by courtesy of Jan Draaistra). DNA of 21 susceptible varieties was available (Gebhardt et al. 2004) from the collection maintained by the IPK potato germplasm bank at Groß-Lüsewitz (Germany). *S. vernei* clone 62-33-3 (Ross 1986) and the interspecific hybrid clone AM78.3778 (Rouppe van der Voort et al. 1998) were kindly provided by Björn Niere (Institute for Nematology, BBA Münster, Germany). Clone AM78.3778 has been originally provided by Richard Janssen.

DNA of the following *Solanum* species was available (Gebhardt et al. 2004); the number of accessions per species is given in parenthesis: *S. acaule* (3), *S. alandiae* (3), *S. andigena* (3), *S. berthaultii* (3), *S. brevicaule* (3), *S. bukasovii* (3), *S. canasense* (3), S. *chacoense* (3), *S. demissum* (3), *S. dulcamara* (1), *S. etuberosum* (2), *S. gourlayi* (3), *S. hondelmannii* (2), *S. kurtzianum* (3), *S. leptophyes* (3), *S. lignicaule* (1), *S. maglia* (1), *S. megistacrolobum* (2), *S. microdontum* (3), *S. morelliforme* (1), *S. nigrum* (1), *S. neorossii* (1), *S. oplocense* (3), *S. phureja* (3), *S. pinnatisectum* (1), *S. sparsipilum* (3), *S. spegazzinii* (3), *S. stenotomum* (3), *S. stoloniferum* (3), *S. vernei* (3), *S. verrucosum* (3). Accession numbers are available from the authors upon request.

Assessment of resistance to *G. pallida*

The genotypes of the Gpa-SR population were tested for resistance to *G. pallida* at the 'Landesamt für Landwirtschaft, Lebensmittelsicherheit und

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Table 1 Population test of markers HC, BA213c14t7-snp139, BA213c14t7-snp274 and SPUD1636 (Bryan et al. 2002) in varieties susceptible or resistant to *G. pallida* according to passport data

Variety or clone	Breeder	Resistance to *G. pallida* pathotypes[a]	HC	snp139	snp274	SPUD1636
Angela	BNA	Susceptible	0	GGGG	AAAA	0
Arkula	NORIKA	Susceptible	0	GGGG	AAAA	0
Assia	Uniplanta	Susceptible	0	GGGG	AAAA	0
Christa	KWS Saat (N: Ragis)	Susceptible	0	GGGG	AAAA	0
Clarissa	BNA	Susceptible	0	GGAA	AAAA	0
Desiree	Lange, W.	Susceptible	0	GGGG	AAAA	0
Gloria	Saatzucht Soltau – Bergen	Susceptible	0	GGGG	AAAA	0
Grata	Stader Saatzucht	Susceptible	0	GGGG	AAAA	0
Hela	Vereinigte Saatzuchten	Susceptible	0	GGGG	AAAA	0
Karat	NORIKA	Susceptible	0	GGAA	AAAA	0
Karlena	NORIKA	Susceptible	0	GGGG	AAAA	0
Koretta	NORIKA	Susceptible	0	GGGG	AAAA	0
Lyra	BNA	Susceptible	0	GGGG	AAAA	0
Maxilla	NORIKA	Susceptible	0	–	–	0
Milva	Saatzucht Berding	Susceptible	0	GGGG	AAAA	Not tested
Nora	BNA	Susceptible	0	GGGG	AAAA	0
Selma	Bavaria Saat	Susceptible	0	AAAA	AAAA	0
Tempora	BNA	Susceptible	0	GGAA	AAAA	0
Toccata	BNA	Susceptible	0	GGAA	AAAA	0
Tomensa	BNA	Susceptible	0	GGGG	AAAA	0
Ute	Bavaria Saat	Susceptible	0	GGGG	AAAA	0
Avano	Karna	Pa2/3	0	AAAA	TTTT	0
Avarna	Karna	Pa2/3	1	AAAA	TTTT	1
Aveka	Karna	Pa2/3	1	GGAA	AATT	1
Averia	Karna	Pa2/3	1	AAAA	TTTT	1
Aviala	Karna	Pa2/3	1	AAAA	TTTT	1
Brisant	Bavaria Saat	Pa2/3	1	GGAA	AATT	0
Festien	E.J. Feunekes	Pa2/3	1	AAAA	AAAA	1
Florijn	Hoiting	Pa2/3	1	GGAA	ATTT	0
Goya	J. Goosen	Pa2/3	1	GAAA	ATTT	0
Innovator	HZPC	Pa2/3	1	AAAA	AAAT	0
Karakter	Averis Saatzucht	Pa2/3	1/0[b]	GGGG	AAAA	0
Kartel	Karna	Pa2/3	1	GAAA	ATTT	1
Melanie	H.K.Kroeze & G.M. Bunte	Pa2/3	1	GGAA	AATT	1
Menco	J.H. Mencke	Pa2/3	1	GGAA	AATT	1
Mercator	J.H. Mencke	Pa2/3	1	GAAA	AATT	0
Mercury	J.H. Mencke	Pa2/3	1	GGAA	AATT	0
Nomade	Matschaap Boerhave VOF	Pa2/3	1	GGAA	AATT	0
Seresta	R.H. Sloots	Pa2/3	1	GAAA	ATTT	1
Sjameto	Agrico	Pa2/3	1	GGAA	AATT	1
Stabilo	R.H. Sloots	Pa2/3	1	AAAA	TTTT	1
Starga	R.H. Sloots	Pa2/3	1	GGAA	AATT	1
Valiant	H. Kuipers	Pa2/3	1	GAAA	TTTT	1
AM78.3778		Pa2/3	1	–	–	1
Elles	B.F. Leestemaker & A. Smid	Pa2	0	GGGG	AAAA	1
Feska	E.J. Feunekes	Pa2	0	AAAA	AAAA	0
Karida	Karna	Pa2	0	GGGG	AAAA	0
Karnico	Karna	Pa2	0	GGGG	AAAA	1
Producent	Kweekbedrijf Prummel	Pa2	0	GGGG	AAAA	0
S.vernei 62-33-3		Pa2	0	–	–	1
Sante	J. Vegter	Pa1/2	0	GGGG	AAAA	1
Amado	BNA	Pa2/3 partial	1	GGAA	AATT	0
Kantara	Karna	Pa2/3 partial	0	–	–	1
Kardent	Karna	Pa2/3 partial	1	GGGA	AATT	1

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Table 1 continued

Variety or clone	Breeder	Resistance to *G. pallida* pathotypes[a]	HC	snp139	snp274	SPUD1636
Katinka	Karna	Pa2/3 partial	0	AAAA	AAAA	1
Pallina	Bavaris Saat	Pa2/3 partial	0	GGGG	AAAA	1

[a] Resistance according to '81e rassenlijst Landbouwgewassen 2006' and 'Beschreibende Sortenliste Kartoffeln 2005'

[b] This variety gave inconsistent results for the HC marker in repeated tests

Fischerei Mecklenburg-Vorpommern' (Rostock, Germany) according to Behringer (1969) and Kort et al. (1977). The plants were inoculated with *G. pallida* population 'Chavornay'. The status of nematode resistance of the varieties was obtained from the variety lists of the Netherlands (81e rassenlijst Landbouwgewassen, 2006, ISSN 0168-7484) and Germany (Beschreibende Sortenliste Kartoffeln, 2005, ISSN 1430-9777).

DNA isolation

Young, healthy potato leaves were harvested, freeze dried and stored in air-tight containers at – 20°C. Total genomic DNA was extracted from 0.3 to 0.4 g freeze dried leave material according to Bormann et al. (2004).

SNP analysis

Amplicons were generated from 50 ng genomic DNA template each of the parents and 44 F1 individuals of population Gpa-SR in 30 µl buffer (20 mM Tris–HCl, pH 8.4, 2.5 mM $MgCl_2$, 50 mM KCl), including 200 nM of each primer (Table 2), 100 µM of dNTP and 0.4–1.0 units *Taq* DNA polymerase (Invitrogen Life Technologies, Freiburg, Germany). Standard cycling conditions were: 4 min initial denaturation at 94°C, followed by 30 cycles of 1 min denaturation at 94°C, 1 min annealing at the appropriate T_m and 1 min extension at 72°C. Reactions were finished by 8 min incubation at 72°C. PCR products were examined for quality on ethidium bromide stained agarose gels. Before sequencing, 10 µl PCR were incubated with 1 unit each of shrimp alkaline phosphatase (Amersham Pharmacia Biotech, Piscataway, USA) and exonuclease I (Invitrogen, Carlsbath, USA) at 37°C for 30 min to remove non-incorporated dNTP's and residual single-stranded DNA. The enzymes were inactivated at 80°C for 15 min. Amplicons were custom sequenced on ABI377 or ABI3700 sequencers (PE Biosystems, Foster City, CA, USA) at the Automated DNA Isolation and Sequencing (ADIS) unit of the Max-Planck Institute for Plant Breeding Research using the dideoxy chain-termination method and ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit. The electropherograms of the individual sequencing readouts (trace files) were visually inspected for segregating SNPs, which were detected by singular overlapping base calling peaks and for InDels, which were detected by overlapping sequencing readouts starting from defined nucleotide positions. SNPs were scored including the allele dosage, using both the Data Acquisition & Data Analysis software DAx7.1 (Van Mierlo Software Consultancy) and manual scoring. The dosage of bi-allelic SNPs in heterozygous tetraploid genotypes (3:1, 2:2 or 1:3) was estimated from the height ratio of overlapping base calling peaks. The SNP scores and the categorized resistance scores (resistant or susceptible) of the 44 F1 individuals were arranged as tabular data. For each SNP marker segregating in the Gpa-SR population, between two and five genotype classes were observed, depending on the parental SNP configuration (for example AAAA, AAAG, AAGG, AGGG and GGGG). Pearson's χ^2 test or Fisher's exact test were used to test the observed SNP genotype classes for significant deviation from the H_0 hypothesis of equal frequency in the two phenotypic categories. Statistic tests were computed with SPSS software (SPSS GmbH Software, München, Germany).

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Allele specific PCR: haplotype *c*

Four primers were designed according to Sarkar and Sommer (1991), Lo et al. (1991) and Okimoto and Dodgston (1996). The two pairs of forward and reverse primers varied at the 3′ end for the BA87d17t3-snp212 alleles *C* or *T* and the snp444 alleles *G* or *A*, respectively. A mismatch was introduced at the third nucleotide from the 3′ end (Okimoto and Dodgston 1996). All four possible primer combinations were tested for specific amplification of haplotype *c* in the Gpa-SR family. The combination of the forward primer 5′ ACACCACCTGTTTGATAAAAAACT 3′ with the reverse primer 5′ GCCTTACT-TCCCTGCTGAAG 3′ resulted in haplotype *c*

Table 2 Targeted loci, oligonucleotides used for PCR amplification and amplicon sequencing, and SNPs/InDels evaluated in resistant and susceptible F_1 plants of the Gpa-SR population

Locus	LG	Oligonucleotide sequences 5′-3′	SNP number[b]	InDel no.[b]
BA114i24t3	I	f-AGATCTGTGGATGTATAGGCGTAG[a] r-CTAACACAATGTACTTGCAGGTG	53, 58, 65, 79	93
St3.2	II	f-AGCAACTTAGGTCACAACCACAC[a] r-TATCTTGAATTGTTTCCCTGCAGC	52, 162	–
St4cl	III	f-AGCTTGTTTTGCGGGGAAAGTG[a] r-AAATGGTCCAATTGTCACTTTATGC	91, 100, 184, 228, 319	–
BA70b11t7	IV	f-ATTCTTGAGATATTGTAACCCAAAC r-TTTGAGGAACATATTAGACTTGTTG fs-CTGATTGGAAGATGATCATAAG[a]	105, 127, 166, 181	–
BA87d17t3	V	f-GTAGTACATCAACATACATTTTGCGG r-CTCAGAATTCAGAGCTTCAACTGATG fs-AACAGGCTTAATCCTCATCCGC[a]	109,**212** ***[c],222, 383, 384, 444	–
BA213c14t7	V	f-CAATTGATTCATTTTATGTAGCGAG r-TCTTGACGCAAACCTCTGCGAG fs-AAATATAAGATATAACTAAATTAA C[a]	96, **139** ***, **152** **, 214, 218, **236** **, 244, 273, **274** ***, 284, **287** **, 289, 305, 345, 347	–
BA76o11t3	V	f-CAGGACATCAATATAAATACTGTTGC r-CGTACGTATGAGGAGTCTGTATC fs-CTATTTGTTCCTTCATGTGTCCTGGA[a]	**336** **	363
BA132h9t3	V	f-GAAAGGCCATGTATATGCAGC r-GCTATCAATATACTTATCTGCTC rs-GTAACTTCTCTTCTTGAGGTGGAC[a]	178, 224, 260, 272, 287	–
BA228g19t7	VII	f-GACTGTTGAGCCAACATGACTC r-TGGAATTATGTTCAGCTTTGGTGA[a]	259, 296, 298, 300, 315, 342, 365, 369, 372, 373	–
BA73e8t3	VIII	f-TGGCCTGAATTTGGATCAAATGG r-GTTAATATCACTCATGGCACTATG fs-CAAATAAAGGTATATGAAGATCTG[a]	107, 117	124
BA261b9t7	VIII	f-TCAAAATTCACAGGGTGATTGGC[a] r-ATGAAGTTACTCAGGCTAACAGG	115, 128, 144, 147, 175, 209, 229, 256, 260, 270	–
GP129	IX	f-GTGGTAGCAAAGTATTCATC[a] r-CGTTATCTGGACTCCTTTAG	55, 94, 148, 149, 161, 178, 190, 196, 205, 211	–
CP105	X	f- GATGTTGTACAAGCTTGTCAAACC[a] r- CAAAATCAGGCCATTGTGAATGAG	181, 203, 237, 276, 284	–
BA81l15t3	X	f- CTGTTGGGTCTTCCTATAAGTTGG[a] r- TGAAACCACTAAACATGACATTTTG	93, 114, 162, 218, 235, 239, 241	–
NL27	XI	f-TAGAGAGCATTAAGAAGCTGC r-TTTTGCCTACTCCCGGCATG rs-AGAGCAGTCCTCCATCCTTTCAC[a]	212, 214, 329, 420, 424, 425, 438, 439, 453, 454	–
			Total 96 SNPs	3 Indels

[a] Primer was used for sequencing the amplicons

[b] SNP and InDel numbering is according to the PoMaMo database (https://www.gabi.rzpd.de/projects/Pomamo/). New SNPs are numbered relative to known flanking SNPs

[c] *** and **: unequal distribution of SNP alleles between resistant and susceptible genotypes, significant at $P < 0.001$ and $P < 0.01$, respectively

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specific amplification of a 276 bp DNA fragment using the following protocol: 50 ng template DNA were amplified in 15 µl PCR-mix (20 mM Tris–HCl, pH 8.4, 1.5 mM $MgCl_2$, 50 mM KCl, 100µM dNTP, 200 nM of each primer, 2% v/v DMSO, 1 unit *Taq* DNA polymerase) and the PCR touchdown conditions: 5 min at 94°C, one cycle of 1 min at 94°C, 1 min at 65°C and 1 min at 72°C, six cycles of 30 s at 94°C, 30 s at 65°C decreasing the annealing temperature to 60°C by 1°C per cycle and 30 s at 72°C, 30 cycles of 30 s at 94°C, 30 s at 60.5°C and 30 s at 72°C, finally 5 min at 72°C. PCR products were separated on standard agarose gels and visualized with ethidium bromide staining. The marker SPUD1636 was amplified as described (Bryan et al. 2002).

Results

Based on evaluating 230 individuals of the Gpa-SR family for resistance to *G. pallida* population 'Chavornay', the 30 most resistant and 30 highly susceptible F_1 individuals were selected for genotyping. Amplicons were generated at 15 loci (Table 2) from genomic DNA of parents SR5 and SR6, 22 resistant and 22 susceptible F_1 plants, and sequenced. In some cases, the use of a nested primer for amplicon sequencing instead of the forward or reverse PCR primer (Table 2) improved the quality of the sequence trace files used for SNP scoring. At this stage, the number of F_1 individuals, of which amplicons were sequenced was limited to 46 for practical reasons (half of a 96-well plate). Between one and fifteen segregating SNPs were scored per amplicon, amounting to 96 scored SNP markers in total (Table 2). Three segregating InDels were also evaluated. Most SNPs have been identified previously in the parental genotypes SR5 and SR6 (Rickert et al. 2003, https://www.gabi.rzpd.de/projects/Pomamo/). However, few new SNPs were also recorded. SNPs and InDels were tested for significant deviation from equal distribution between the two groups of highly resistant and susceptible genotypes by the χ^2 test. Seven SNP markers deviated significantly ($P < 0.01$) from equal distribution (Table 2), indicating linkage to *G. pallida* QRL. All seven significant SNPs were located in the amplicons generated from three BAC insertion ends that are part of a local physical map on potato chromosome V in the interval between marker loci GP21 and GP179, which includes the *R1* gene for resistance to *Phytophthora infestans* (Ballvora et al. 2002, Ballvora et al. manuscript in preparation). The position of the 250 kbp contig within the GP21-GP179 interval, the physical distances between the amplicons and the positions of the significant SNPs are shown in Fig. 1.

The seven SNP markers linked to a *G. pallida* QRL fell into two groups of tightly linked or co-segregating SNPs. The first group included three SNP markers: snp212 in amplicon BA87d17t3, snp139 and snp274 both in amplicon BA213c14t7. The second group consisted of four SNP markers: snp336 in amplicon BA76o11t3, snp152, snp236 and snp287 in amplicon BA213c14t7. A genetic model based on three different haplotypes *a*, *b* and *c* (Table 3) best explained the observed SNP genotype classes of the seven significant SNP markers in parents SR5 and SR6, and in 43 F1 hybrids (amplicon sequencing of one susceptible genotype consistently failed). 268 (91%) of 294

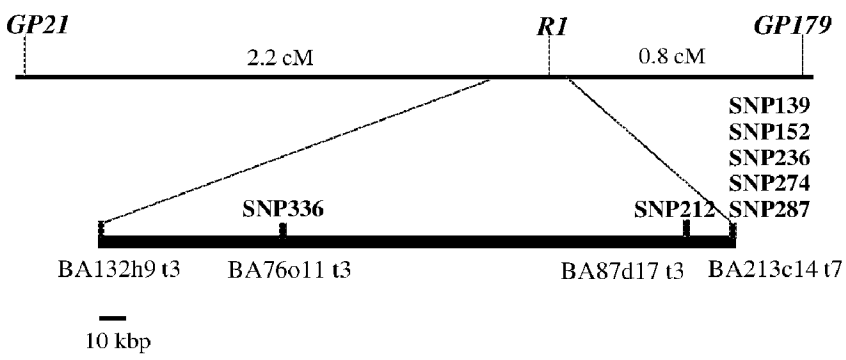


Fig. 1 Positions of the seven SNP markers linked to the major QTL for resistance to *G. pallida* on the partial physical map in the interval GP21–GP179 on potato chromosome V

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Table 3 Haplotype model deduced from the observed segregation of seven SNP markers linked to QTL for nematode resistance on chromosome V in the SR-Gpa family. Haplotype specific nucleotides are shown in bold letters

Haplotype	BA76o11t3	BA87d17t3	BA213c14t7				
	snp336	snp212	snp287	snp274	snp236	snp152	snp139
a	C	C	T	A	C	T	G
b	**G**	C	**C**	A	**T**	**A**	G
c	C	**T**	T	**T**	C	T	**A**

SNP scores (7 SNPs scored in 43 genotypes with few missing data) were in agreement with this haplotype model. The remaining scores (9%) showed a discrepancy with the model, most likely resulting from ambiguous scores of the allele dosage. According to the model, the resistant parent SR6 was duplex for haplotypes *a* and *c* (*aacc*) and the susceptible parent SR5 was duplex for haplotypes *a* and *b* (*aabb*). When assuming tetrasomic inheritance and random formation of 2n gametes in both parents, nine genotype classes are expected in the F_1 progeny in the frequencies shown in Table 4. Only five genotype classes were actually observed in the 43 examined F_1 individuals, of which four deviated significantly from the expected numbers (Table 4). The genotype class expected to be the most frequent (*aabc*) was completely absent in the progeny. Under the alternative hypothesis of disomic inheritance, four genotype classes with equal frequency are expected in F_1. In fact, three of the five observed genotype classes fitted the model of disomic inheritance, whereas the forth expected class (*bbcc*) was not observed and the observed classes *aaac* and *abbc* were not expected (Table 4). The resistant parent SR6 and all 22 highly resistant F_1 individuals carried haplotype *c* either in duplex or simplex condition. With the exception of one individual, all susceptible F_1 genotypes lacked haplotype *c*, indicating strong linkage between haplotype *c* and a nematode resistance allele descending from SR6 (Table 4). Haplotype *b* descending from the susceptible parent SR5 was more frequent in susceptible (13) than in resistant F1 individuals (9). Haplotype *b* was therefore linked to a susceptibility allele. Nine of the ten individuals having both haplotypes *c* and *b* were highly resistant, indicating that the nematode resistance allele linked to haplotype *c* was mostly dominant (Table 4).

A PCR assay was developed to detect specifically haplotype *c*, based on the SNP marker BA87d17t3-snp212, which had the haplotype *c* specific allele *T* (Table 3) and on snp444 (*T*/*C*) in the same amplicon (Table 2). Snp444 was arbitrarily chosen for its physical distance from snp212, allowing the generation of a PCR product of suitable length. Only the primer combination specific for snp212-T/snp444-G amplified a 274 bp fragment in the resistant parent SR6, in all 30 highly resistant F_1 individuals selected and in the single, susceptible individual having haplotype *c* (Fig. 2). The susceptible parent SR5 and 29 susceptible F_1 individuals were negative for the haplotype *c* specific PCR product, which is subsequently referred to as the HC marker.

Thirty four varieties with resistance to *G. pallida* pathotypes Pa2 or Pa2/Pa3 according to passport data, the differential *S. vernei* clone 62-33-3 (Pa2) (Ross 1986) and AM78.3778 (Pa2/3) (Rouppe van der Voort et al.1998) and 22 susceptible varieties as control group were tested for presence of the HC marker. In addition, the 56 varieties were scored for the haplotype *c* specific SNP markers BA213c14t7-snp139 and snp274 by amplicon sequencing (Table 1). With the exception of cvs 'Avano' and 'Karakter', all varieties with high resistance to *G. pallida* pathotype Pa2/3 and clone AM78.3778 were HC positive, whereas the six varieties with resistance to pathotype Pa2 only, the *S. vernei* clone 62-33-3 and all susceptible varieties were HC negative (Table 1). From five varieties with reported intermediate or partial resistance to *G. pallida* (Amado, Kantara, Kardent, Katinka and Pallina) two were HC positive and three were negative (Table 1). These data indicated that the HC marker was highly diagnostic for the presence of an allele that confers high levels of resistance to *G. pallida* pathotype Pa2/3. The haplotype *c* specific allele *T* of

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Table 4 Genetic models for the Gpa-SR population based on haplotypes *a*, *b* and *c* and distribution of nematode resistant and susceptible plants in the observed genotype classes

	Genotype	Observed genotype numbers $N = 43$	Expected genotype numbers, tetrasomic inheritance $N = 43$	χ^2 test for deviation from expected numbers; tetrasomic inheritance	Expected genotype numbers, disomic inheritance $N = 43$	χ^2 test for deviation from expected frequencies; disomic inheritance	No. of resistant genotypes	No. of susceptible genotypes
SR5	*aabb*						resistant	susceptible
SR6	*aacc*							
F1	*aaaa*	8	1.19	$P < 0.005$	10.75	n.s.	0	8
	aabb	11	1.19	$P < 0.005$	10.75	n.s.	0	11
	aacc	13	1.19	$P < 0.005$	10.75	n.s.	13	0
	bbcc	0	1.19	n.s.	10.75	$P < 0.005$	0	0
	aaac	0	4.76	$P < 0.05 > 0.025$	0	–	0	0
	aaab	1	4.76	n.s.	0	–	0	1
	abbc	10	4.76	$P < 0.025 > 0.01$	0	–	9	1
	abcc	0	4.76	$P < 0.05 > 0.025$	0	–	0	0
	aabc	0	19.11	$P < 0.005$	0	–	0	0
							$\Sigma = 22$	$\Sigma = 21$

snp274 (Table 3) was in nearly complete linkage disequilibrium with the HC marker, the only exceptions being cvs 'Avano' and 'Festien' (Table 1). Based on the dosage of snp274 allele *T*, the *G. pallida* resistant varieties were mostly duplex, triplex or even quadruplex for the resistance allele. In contrast, the haplotype *c* specific allele *A* of snp139 (Table 3) was less diagnostic, as it occurred in all varieties with resistance to *G. pallida* pathotype Pa2/3, but also in 5 susceptible varieties and in cv 'Feska' being resistant only to pathotype Pa2 (Table 1).

A DNA collection of 31 *Solanum* species was screened for presence of the HC marker. HC was exclusively detected in accessions of *S. vernei* (data not shown), identifying *S. vernei* as the most likely source of the resistance to *G. pallida* pathotype Pa2/3.

The set of potato varieties and *S. vernei* differential clones was tested for presence or absence of the diagnostic SPUD1636 marker, which is linked to a major QRL to *G. pallida* in the same region on potato chromosome V (Bryan et al. 2002). All susceptible varieties tested lacked the SPUD1636 marker, but SPUD1636 was present in only 12 of the 22 highly resistant varieties, in 8 of the 12 varieties with incomplete resistance and in both clones *S. vernei* 62-33-3 and AM78.3778 (Table 1). SPUD1636 was therefore less diagnostic for high resistance to *G. pallida* pathotype Pa2/3 than the HC marker, BA213c14t7 snp139 and snp274.

Discussion

SNP genotyping by sequence analysis of heterozygous amplicons

The cultivated, tetraploid potato is highly polymorphic and heterozygous at the DNA sequence level, allowing parallel and quantitative scoring of several SNP markers in sequence trace files of single amplicons (Rickert et al. 2003). This notion was confirmed when genotyping the Gpa-SR family for 96 SNP markers in 15 amplicons. Most, but not all SNP positions and SNP allele dosages were reproducible when comparing the SNP's segregating in the Gpa-SR family with the SNP's scored in the genotype panel of Rickert et al.

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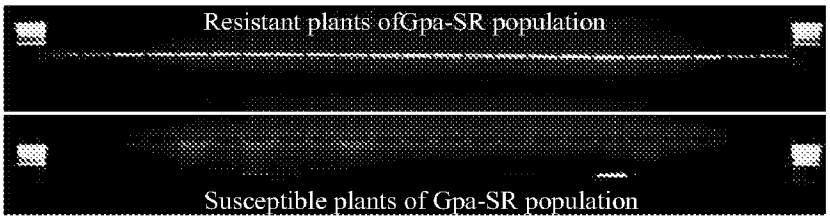


Fig. 2 Distribution of the HC marker in the highly nematode resistant (top) and susceptible (bottom) subpopulations selected from the SR-Gpa family

(2003), which included the parents SR5 and SR6. Some new SNP's were also detected in the Gpa-SR family. Moreover, genetic modeling uncovered discrepancies between 9% of the individual SNP scores and the genetic model. These inconsistencies and discrepancies point to scoring errors, particularly of the SNP allele dosage. The peak height in the sequence trace files used as measure for the allele dosage can be influenced differently for different nucleotides by the surrounding sequence context. Unequal allele amplification in heterozygous genotypes can also lead to erroneous assessment of the allele dosage. Generating very high quality sequences from locus specific, uniform amplicons can minimize, but does not eliminate completely these problems. The sequence quality of some amplicons could be improved by using for sequencing a nested primer located in an apparently 'SNP free' region of the sequence. The locus specificity of the amplicons depends on the redundancy of the genomic sequence and had to be determined experimentally case by case.

Distorted segregation

The pedigree information of the Gpa-SR family made possible to identify three haplotypes *a, b* and *c*, based on the observed segregation patterns of seven SNP markers significantly linked to a major QTL for resistance to *G. pallida*. The haplotypes were used to assign a genotype to each parent and to 43 F1 individuals. The observed genotype classes and the observed number of individuals in each genotype class fitted neither the model of tetrasomic nor disomic inheritance. Genotype classes expected for either model of inheritance were completely absent in the progeny, and frequencies of observed genotype classes strongly deviated from expectation. These distorted frequencies of genotype classes can be the result of an intermediate mode of inheritance with preferential pairing of homologous chromosomes due to structural differences between different haplotypes and/or of the strong selection applied for highly resistant versus highly susceptible individuals. The absence of certain genotype classes (e. g. *aabc* in tetrasomic inheritance or *bbcc* in disomic inheritance) implicates incomplete nematode resistance of these classes. This may indicate interactions between different alleles at the linked nematode resistance locus and incomplete dominance of the resistance allele linked to haplotype *c* in certain allele constellations. This observation is compatible with the detection of haplotype *c* in some varieties with incomplete resistance to *G. pallida* pathotype Pa2/3.

Position of the nematode resistance gene linked to SNP haplotype *c*

Knowledge on the distribution of loci for pathogen resistance in the potato genome (Gebhardt and Valkonen 2001, https://www.gabi.rzpd.de/projects/Pomamo/) facilitated the mapping of the unknown nematode resistance locus in the Gpa-SR population by selective genotyping with SNP markers. Probing only twelve regions on ten potato chromosomes was sufficient to map the resistance locus. All SNP markers that showed significant linkage with resistance were located in the interval GP21–GP179 on potato chromosome V, in a physical region containing a family of NB-LRR type genes, one member of which has been functionally characterized as the *R1* gene for resistance to late blight (Ballvora et al. 2002; Kuang et al. 2005), whereas no function is known for the other members of the family. One or more members of the *R1* gene family are good positional candidates for being the molecular basis also of the genes for nematode resistance located

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in this region, similarly as has been found for genes *Rx1* for resistance to *Potato Virus X* (PVX) and *Gpa2* for resistance to *G. pallida* pathotype Pa2, which are both highly homologous members of a clustered NB-LRR type gene family on potato chromosome XII (van der Vossen et al. 2000). To the same genomic region on potato chromosome V as identified in this paper, major QTL for resistance to *G. pallida* and *G. rostochiensis* have been mapped previously, which originated from the wild species *S. spegazzinii*, *S. vernei*, *S. sparsipilum* and possibly others (Kreike et al. 1994; Rouppe van der Voort et al. 1998, 2000; Bryan et al. 2002; Caromel et al. 2003; 2005). *S. vernei* is the most likely source of the resistance allele present in breeding clone SR6 and in most varieties with high resistance to *G. pallida* pathotype Pa2/3. This was shown when a panel of 31 *Solanum* species was screened for presence of the haplotype *c* specific HC marker and only *S. vernei* accessions were found to be HC positive. The major locus for resistance to *G. pallida* detected in the Gpa-SR population is therefore identical or very similar to the major cyst nematode QRL *Grp1* and *Gpa5* described previously (Rouppe van der Voort et al.1998, 2000). The resistance allele of *S. vernei* detected by the HC marker on potato chromosome V is named *RGp5-vrnHC*.

The diagnostic value of the HC nematode resistance marker

The HC marker is the most diagnostic DNA marker currently available for potato resistance to *G. pallida*. It was detected in almost all tested German or Dutch potato varieties with high resistance levels to *G. pallida* pathotype Pa2/3. This indicates that the allele *RGp5-vrnHC* has been widely used in resistance breeding at least in Germany and the Netherlands. The origin of this allele is unknown, as several *S. vernei* clones have been described as resistance sources (Ross 1986), which are not available any more for testing. At this point, we can only exclude as source clone *S. vernei* 62-33-3, which is resistant to *G. pallida* pathotype Pa2 (Ross 1986) and was accordingly HC negative. However, *S. vernei* accessions available in germ plasm collections can now easily be screened by PCR with the HC marker. HC positive plants may also be highly resistant to *G. pallida*. We are currently testing whether the HC marker retains its diagnostic value for resistance to *G. pallida* pathotype Pa2/3 in different accessions of *S. vernei*. Information on presence or absence of the HC marker in parental clones or advanced breeding material will be useful in breeding programs. Knowing the allele dosage provides additional information on the expected transmission frequency of the resistance. The allele dosage cannot be assessed by the qualitative PCR test for the HC marker. However, other high throughput SNP detection methods such as pyrosequencing are suitable for allele quantification (Rickert et al. 2002) and could be applied to the most diagnostic SNPs BA87d17t3-snp212 and BA213c14t7-snp274. The HC marker can also be helpful for discriminating among different field populations of *G. pallida*, by using HC positive and HC negative genotypes as genetically precisely characterized differentials.

There are a few limitations to the predictive power of the HC marker. Variety 'Avano', which is highly resistant to *G. pallida* Pa2/3 according to passport data, was HC negative. In this case, a recombination event might have separated the HC marker from the resistance allele *RGp5-vrnHC*, or 'Avano' has a different source of resistance, or the resistance assessment might be ambiguous. On the other hand, some varieties with incomplete resistance such as 'Amado' and 'Kardent' were HC positive. This can result from incomplete dominance of the *RGp5-vrnHC* allele in certain genetic backgrounds or from resistance assessment using *G. pallida* populations, which partially overcome the resistance allele *RGp5-vrnHC*.

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Gene *RB* cloned from *Solanum bulbocastanum* confers broad spectrum resistance to potato late blight

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Late blight, caused by the oomycete pathogen *Phytophthora infestans*, is the most devastating potato disease in the world. Control of late blight in the United States and other developed countries relies extensively on fungicide application. We previously demonstrated that the wild diploid potato species *Solanum bulbocastanum* is highly resistant to all known races of *P. infestans*. Potato germplasm derived from *S. bulbocastanum* has shown durable and effective resistance in the field. Here we report the cloning of the major resistance gene *RB* in *S. bulbocastanum* by using a map-based approach in combination with a long-range (LR)-PCR strategy. A cluster of four resistance genes of the CC-NBS-LRR (coiled coil–nucleotide binding site–Leu-rich repeat) class was found within the genetically mapped *RB* region. Transgenic plants containing a LR-PCR product of one of these four genes displayed broad spectrum late blight resistance. The cloned *RB* gene provides a new resource for developing late blight-resistant potato varieties. Our results also demonstrate that LR-PCR is a valuable approach to isolate genes that cannot be maintained in the bacterial artificial chromosome system.

Potato late blight, a disease caused by the oomycete pathogen *Phytophthora infestans*, is one of the world's most devastating plant diseases. Late blight was responsible for the European potato famine in the 19th century, which caused the starvation deaths of more than one million people in Ireland alone. Despite its historic significance, none of the currently grown potato cultivars in the United States have adequate late blight resistance. Currently, late blight is responsible for multibillion-dollar losses annually in both potato and tomato production (1). Furthermore, in developing countries, where funds for purchasing fungicides are limited, late blight can completely eliminate the potato crop.

A number of wild potato species, such as *Solanum demissum* ($2n = 6x = 72$), coevolved with *P. infestans*, and have provided the primary germplasm for breeding late blight resistance in cultivated potato. At least 11 resistance (R) genes that originated from *S. demissum* have been incorporated into various potato cultivars (2). All of these 11 R genes confer race-specific hypersensitive resistance. Potato cultivars possessing such R genes are not resistant to all races of the pathogen. These race-specific R genes provide only short-lived resistance in the field as new virulent races of the pathogen rapidly overcome the resistance encoded by single race-specific resistance genes (3, 4).

A wild diploid potato species, *Solanum bulbocastanum* ($2n = 2x = 24$), is highly resistant to all known races of *P. infestans*, even under intense disease pressure (5). Because *S. bulbocastanum* is sexually incompatible with potato, somatic hybrids between potato and this wild species were developed with the long-term goal of capturing this resistance for use in potato cultivars. The somatic hybrids and a number of backcrossed progenies consistently displayed late blight resistance similar to the parental *S. bulbocastanum* clone PT29 (5, 6). Unlike potato varieties containing the R genes derived from *S. demissum*, no obvious necrotic lesions, which are characteristic of the classical hypersensitive response, were observed. In fact, the pathogen sometimes sporulates on PT29-derived resistant materials. The resistance of the PT29-derived plants is manifested as a slow progression of lesion development that substantially decreases the rate of disease development in the plants. This phenotype of general suppression but not elimination of symptom development has been consistently observed in field tests at various locations in the United States and in Toluca, Mexico, between 1995 and 2002. The late blight resistance associated with the PT29-derived materials could be considered rate-reducing or, in some terms, partial resistance, and can be effectively used in resistance breeding programs (7).

Several populations have been developed from a fertile somatic hybrid between potato and *S. bulbocastanum* clone PT29 and its backcrossed progenies. A major resistance locus, *RB*, was mapped to a specific location on chromosome 8 of *S. bulbocastanum* (8). A bacterial artificial chromosome (BAC) contig spanning the *RB* gene was constructed by using a reiterative approach of BAC walking and high-resolution genetic mapping (9). In this paper, we report the cloning of the *RB* gene by a map-based approach used in combination with long-range (LR)-PCR. The cloned *RB* gene will provide an important resource to develop late blight-resistant potato cultivars and further understand disease resistance mechanisms in plants.

Materials and Methods

DNA Sequencing and Analysis. The DNA sequences of the *S. bulbocastanum* BAC clones 177O13 and CB3A14 were determined by using a shotgun sequencing strategy as described by Yuan *et al.* (10). The two BACs were deposited in the PLN division of GenBank [accession nos. AY303171 (177O13) and AY303170 (CB3A14)]. Multiple sequence alignments were conducted by using CLUSTALX 1.81 software (11). Diversifying selections were investigated by using PAML (12, 13).

Long-Range PCR. Primers were designed for each candidate *RB* gene (from the resistant *RB* haplotype) based on the sequence information of BAC 177O13 (the susceptible *rb* haplotype). Individual candidate *RB* genes were amplified by LR-PCR, using genomic DNA of *S. bulbocastanum* clone PT29 as a template. Eight-base pair sequences (CGGGATCC) were introduced into all LR-PCR primers (Table 3, which is published as supporting information on the PNAS web site, www.pnas.org) to create a *Bam*HI restriction site to facilitate cloning of the LR-PCR products. LR-PCR reactions were carried out in a total volume of 50 μl containing 2.5 units of Takara LA *Taq* (PanVera, Madison, WI), 1× reaction buffer, 400 μM dNTP, 100 ng of

Abbreviations: BAC, bacterial artificial chromosome; LR-PCR, long-range PCR; R, resistance; RGA, R-gene analog.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. AY303171 and AY303170).

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Table 1. *P. infestans* isolates used in late blight resistance evaluation

Isolate	Genotype	Mating type	Race	Source
US930287	US-8	A2	ND	William Fry, Cornell University, Ithaca, NY
126C18	US-8	A2	0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11	Neil Gudmestad, North Dakota State University
US940480	US-8	A2	0, 1, 2, 3, 4, 5, 6, 7, 9, 10, 11	William Fry, Cornell University
MSU-96	US-8	A2	0, 1, 2, 3, 4, 5, 6, 7, 8, 10, 11	Ken Deahl, U.S. Department of Agriculture–Agricultural Research Service, Beltsville, MD
US940501	US-1	A1	0, (6)*	William Fry, Cornell University
US980008	US-11	A1	ND	William Fry, Cornell University

ND, not determined.

*Parentheses denote variable results; i.e., both compatible and incompatible reactions were observed in different experiments (William Fry, personal communication).

template DNA, and 0.2 μM each primer. The PCR conditions were 1 min at 94°C followed by 35 cycles of 20 sec at 94°C, 20 sec at 60°C, and 15 min at 68°C. The LR-PCR products from three independent reactions were cloned into the pGEM-T or pCR-XL-TOPO vectors. The haplotype origin (*RB* or *rb* haplotype) of the cloned LR-PCR fragments was determined by cleaved amplified polymorphic sequence markers (Table 4, which is published as supporting information on the PNAS web site) developed for fine mapping (9). The final LR-PCR products from the *RB* haplotype were recloned into the *Bam*HI site of the binary cosmid vector pCLD04541 (14) for use in potato transformation.

Complementation Analysis. The pCLD04541 constructs containing putative *RB* genes derived from LR-PCR and BAC CB3A14 were introduced into *Agrobacterium tumefaciens* strain LBA4404 by electroporation. Potato (*Solanum tuberosum* cv. Katahdin) transformation was performed essentially as described by Ziegelhoffer *et al.* (15). Kanamycin-resistant transgenic plants were tested by PCR, using both gene-specific primers and CAPS markers. Plants with confirmed insertion of the putative genes were clonally propagated *in vitro* and screened for late blight resistance. The greenhouse resistance assay was carried out in triplicate by inoculating plants with sporangial suspensions of different isolates of *P. infestans* (Table 1) according to Naess *et al.* (8). Foliage blight scores were recorded at 3, 4, 5, 7, and 10 days after inoculation. The ratings and ranges of percentage infections associated with the scores were as follows: 9, no visible infection; 8, <10%; 7, 11–25%; 6, 26–40%; 5, 41–60%; 4, 61–70%; 3, 71–80%; 2, 81–90%; 1, >90%; 0, 100%.

Rapid Amplification of cDNA Ends (RACE). Three-week-old *S. bulbocastanum* PT29 plants were inoculated with *P. infestans* isolate US930287 and maintained in greenhouses. Equal amounts of challenged and unchallenged leaves at 12 h and 1, 2, 3, 4, and 5 days after inoculation were collected and pooled. Total RNA was isolated from the combined materials by using TRIzol (Invitrogen). Poly(A)$^+$ RNA was isolated by using PolyATract mRNA Isolation Systems (Promega). The 5′ and 3′ ends of the cDNA were determined by RACE, using the GeneRacer kit (Invitrogen). The RACE primers (Table 5, which is published as supporting information on the PNAS web site) are identical between the resistant and susceptible haplotypes.

Results

Cloning of the *RB* Gene by LR-PCR. The *RB* locus was previously mapped to chromosome 8 of *S. bulbocastanum* (8). The *RB* locus is heterozygous (*RB*/*rb*) in the original *S. bulbocastanum* clone PT29 used in the development of genetic mapping populations and BAC libraries. Surprisingly, all 11 BACs associated with the *RB* locus were derived from the *rb* haplotype (9). One of these 11 BACs, 177O13, was fully sequenced. BAC 177O13 contains 163,635 bp, and includes one truncated and four complete CC-NBS-LRR (coiled coil–nucleotide binding site–Leu-rich repeat)-class R-gene analogs (RGAs) (Fig. 1). The one truncated and four complete RGAs in 177O13 are named *rga-tr*, *rga1*, *rga2*, *rga3*, and *rga4*, respectively.

We used a LR-PCR-based approach to clone the RGAs from the *RB* haplotype. Four primer sets (Table 3) were designed based on the sequences in BAC 177O13 to amplify each of the four complete RGAs. DNA isolated from *S. bulbocastanum* clone PT29 was used as the LR-PCR template. Four LR-PCR products with expected sizes of 13.0, 8.6, 11.8, and 7.9 kb were successfully amplified from *S. bulbocastanum* and cloned into the pGEM-T or pCR-XL-TOPO vectors. End sequencing and CAPS markers (Table 4) were used to determine the *rb* or *RB* origin of each cloned LR-PCR fragment. To avoid potential PCR artifacts, we cloned three independent LR-PCR products for each of the four RGAs. All four RGAs derived from both *rb* and *RB* haplotypes were recovered from the LR-PCR experiments. The four RGAs derived from the *RB* haplotype are denoted *RGA1*-PCR, *RGA2*-PCR, *RGA3*-PCR, and *RGA4*-PCR, and were cloned into the *Bam*HI site of the binary vector pCLD04541 for complementation studies.

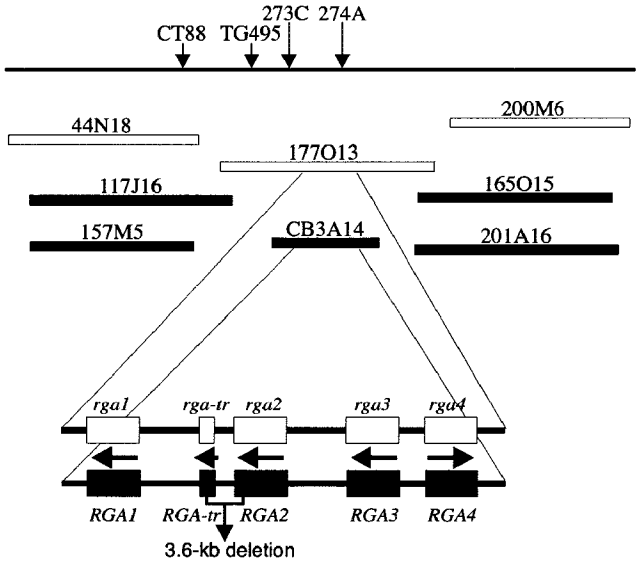


Fig. 1. Genetic and physical maps of the genomic region containing *RB*. The *RB* locus is closely linked to markers CT88, TG495, 273C, and 274A (8, 9). BAC clones from the *RB* haplotype are represented as filled boxes, and BAC clones from the *rb* haplotype are represented as open boxes. Both 177O13 and CB3A14 contain one truncated and four complete RGAs. The direction of transcription of each gene is indicated by an arrow. The 3.6-kb deletion region between *RGA2* and *RGA-tr* is marked. This 3.6-kb fragment is not present in BAC CB3A14 but is drawn for the purpose of comparison with BAC 177O13.

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Table 2. Late blight screening of transgenic plants by using isolate US930287

Construct in transgenic Katahdin	Number of plants tested*	Number of plants showing resistance
RGA1-PCR	13	0
RGA2-PCR (*RB*)	14	14†
RGA3-PCR	21	0
RGA4-PCR	18	0
RGA1-BAC	13	0
RGA2-BAC	11	0
RGA3-BAC	20	0
RGA4-BAC	26	0
Katahdin control	8	0
S. bulbocastanum (PT29) control	6	6

Plants were inoculated with *P. infestans* US930287 and scored for symptoms 7 days after inoculation. Plants were scored as resistant (R) if the resistance score was ≥7.0 (≤25% infection); plants were scored as susceptible (S) if the resistance scores was ≤6.9 (>25% infection).

*These correspond to independent transformation events.

†Of the 14 resistant plants, nine plants had a score of 7 and five plants had a score of 8.

Complementation Analysis of the LR-PCR-Derived RGA Clones. The four RGA clones amplified from the *RB* haplotype were transferred into *A. tumefaciens* strain LBA4404. Katahdin, a potato variety susceptible to late blight, was used for *Agrobacterium*-mediated transformation. Transgenic Katahdin plants (Table 2) developed from the four RGA constructs were inoculated with isolate US930287 of *P. infestans*. Katahdin plants containing *RGA1*-PCR, *RGA3*-PCR, and *RGA4*-PCR, as well as untransformed controls, were susceptible to late blight (Fig. 2 and Table 2). Leaves of all susceptible plants displayed typical spreading lesions, with water-soaked areas and extensive rotting developed at later time points. In contrast, all 14 plants derived from independent transformations by using the *RGA2*-PCR construct displayed resistance to the pathogen. *P. infestans* infection of these plants was significantly delayed and limited to the lower leaves. Small lesions that developed on the lower leaves were restricted to the infected area.

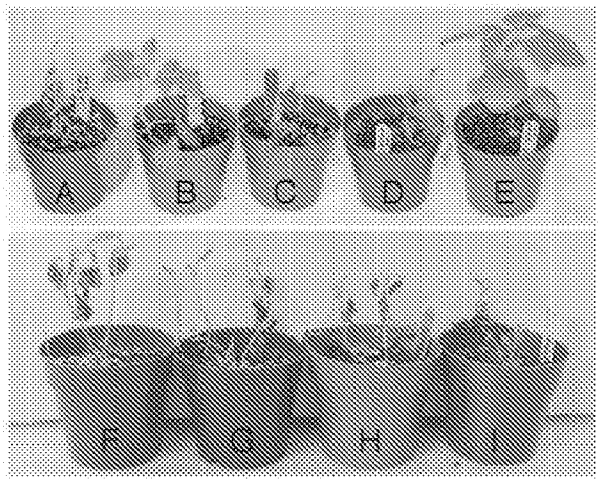


Fig. 2. Complementation analysis of putative *RB* genes. Transgenic Katahdin and control plants were inoculated with the US930287 isolate of *P. infestans*. Disease symptoms were recorded 7 days after inoculation. (*A–C*) Transgenic Katahdin plants containing constructs *RGA1*-PCR, *RGA2*-PCR, and *RGA4*-PCR, respectively. (*D*) Control Katahdin plant. (*E*) Katahdin plant that was not inoculated. (*F–I*) Transgenic Katahdin plants containing constructs *RGA1*-BAC, *RGA2*-BAC, *RGA3*-BAC, and *RGA4*-BAC, respectively.

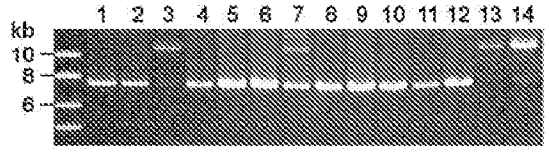


Fig. 3. Stability analysis of CB3A14. Culture from the original well of BAC CB3A14 was streaked, and plasmid DNA from 12 independent clones (lanes 1–12) was isolated and used as a template for PCR analysis with primers GAP-1a and GAP-1b (Table 3) that span the 3.6-kb deletion (Fig. 1). In the experiment represented by lane 13, genomic DNA from *S. bulbocastanum* clone PT29 was used as a template. In the experiment represented by lane 14, DNA from BAC 177O13 was used as a template.

One of the *RGA2*-PCR transgenic lines, SP922, was subsequently tested by using five additional isolates of *P. infestans* (Table 1). The SP922 plants showed remarkable resistance to all five isolates, including 126C18, a "super race" that overcomes all 11 major R genes identified in *S. demissum* (16). Untransformed Katahdin plants were all susceptible (data not shown). SP922 was susceptible to early blight when it was inoculated with isolate AS98-100 of the early blight pathogen *Alternaria solani* (data not shown). This result is consistent with independent segregation of early blight resistance and the *RB* locus observed in BC1 populations derived from the potato–PT29 somatic hybrids (J.P.H., unpublished work). The complementation results demonstrated that *RGA2* represents the functional *RB* gene.

Attempts to Clone *RB* from BAC. Sequence analysis of BAC 177O13 revealed that a 102-kb region, which spans *rb*, is devoid of *Bam*HI restriction sites. Because approximately two-thirds of the PT29 BAC library was developed from partial *Bam*HI digestion (9, 17), the lack of BAC clones containing the *RB* allele may be due to the lack of *Bam*HI sites in this region. We constructed a BAC library, consisting of 8,448 clones, by complete *Bam*HI digestion of PT29 DNA. Four BAC clones were identified by using probes located in the *RB* region. Three of the four clones were derived from the *rb* haplotype. The fourth BAC, CB3A14, was derived from the *RB* haplotype.

Complete sequencing of CB3A14 revealed four complete RGAs. The truncated RGA found in 177O13 was not observed in CB3A14 (Fig. 1). The four RGAs in CB3A14 were designated *RGA1*-BAC, *RGA2*-BAC, *RGA3*-BAC, and *RGA4*-BAC. A cosmid library was developed from CB3A14 by using the binary vector pCLD04541. Cosmid clones containing each of the four RGAs were selected by PCR, using two sets of primers designed for each RGA (Table 6, which is published as supporting information on the PNAS web site), and were used to transform Katahdin. Surprisingly, transgenic Katahdin plants containing all four constructs, including *RGA2*-BAC, did not show increased resistance compared with the control Katahdin plants (Table 2 and Fig. 2). These results indicate that *RGA2*-PCR and *RGA2*-BAC do not represent the same genomic DNA sequences. Sequence analyses of *RGA2*-PCR and *RGA2*-BAC, as well as BACs 177O13 and CB3A14, revealed that a 3.6-kb deletion occurred in CB3A14 between the 3′ region of *RGA2* and the middle of *RGA-tr* (Fig. 1).

A pair of primers (Table 3) spanning the putative 3.6-kb deletion were designed based on the CB3A14 sequence. An expected 7-kb fragment was amplified from CB3A14, whereas a 10-kb fragment was amplified from both PT 29 genomic DNA and BAC177O13 (Fig. 3). We then conducted PCR analysis of 12 independent clones from the original well containing BAC CB3A14. A 7-kb band was amplified from 10 of the 12 clones.

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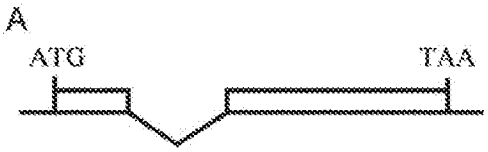
One clone (Fig. 3, lane 7) produced both 7- and 10-kb products, indicating that it is likely a mixture of the deletion derivatives and intact fragments. A second clone (Fig. 3, lane 3) produced a major 10-kb band and a few faint smaller bands, similar to the pattern produced from PT29 genomic DNA. We were unable to rescue this clone by retransforming the BAC plasmid into different *E. coli* strains because a similar deletion pattern was again observed. These results confirmed that the original BAC CB3A14 cannot be stably maintained in *E. coli*.

Transcript Analysis of the RGAs. The structures of the four RGAs were determined by comparing the genomic DNA sequences derived from BAC CB3A14 and LR-PCR products with 5′ and 3′ RACE products. Multiple RACE products from each experiment were sequenced to avoid PCR errors. Sequences of the 5′ and 3′ RACE products for each RGA overlapped and provided complete coverage of the transcribed region. RACE analysis revealed that *RGA1*, *RGA2* (*RB*), *RGA3*, and *RGA4* are all expressed and have a similar structure with a single intron (Fig. 4*A*). *RB* was transcribed in leaves of *S. bulbocastanum* plants in the absence of pathogen challenge, suggesting that it is constitutively expressed.

Sequences of 5′ and 3′ RACE products of *RGA2* (*RB*) revealed two complete primary transcripts. One corresponded to *rga2* (*rb*) in BAC 177O13 and the other matched with the *RGA2*-PCR sequence except for a 679-bp intron (positions 430–1,108) and three nucleotide mismatches. The three mismatches were caused by LR-PCR, which was confirmed by partial sequencing of the corresponding region of two additional independent LR-PCR products. The 5′ transcript was identical to only the first 2,295 bp of the 5′ sequence of *RGA2*-BAC, excluding an identical intron region observed in *RGA2*-PCR. However, none of the three 3′ transcripts matched the *RGA2*-BAC sequence, confirming that the 3.6-kb deletion starts from the 3′ coding region of *RGA2* (*RB*).

Structure and Evolution of the *RB* Gene Family. The *RB* transcript was determined to be 3,319 bp and to contain 130- and 276-bp UTR at the 5′ and 3′ ends, respectively. The *RB* gene encodes a predicted polypeptide of 970 aa with a molecular weight of 110.3 kDa (Fig. 4*B*). The predicted RB protein belongs to the NBS-LRR class of R proteins (18). Its putative NBS domain consists of three motifs: kinase 1a or P-loop (positions 182–190), kinase 2 (positions 255–264), and kinase 3a (positions 288–293) (Fig. 4*B*). Downstream of the kinase motifs is a domain with unknown function conserved among resistance genes: QLPL, CFAY, and MHD motifs (19). The deduced RB protein contains one putative five-heptad leucine zipper motif near the N terminus (positions 10–45). Another region containing four-heptad repeats (positions 588–609) was observed within the LRR domain (Fig. 4*B*). The LRR domain consists of 21 LRR repeats, several of them imperfect.

The *RB* gene family includes one truncated and four complete genes in both the resistant and susceptible haplotypes (Fig. 1). In the susceptible haplotype, gene *rb* had a point mutation resulting in a premature stop codon (at the 454th codon). Both *rga1* and *rga3* had a 1-bp frame-shift deletion. Therefore, *rb*, *rga1*, and *rga3* are likely to be pseudogenes. The four complete genes in both haplotypes are similar in length (2,895–2,979 bp) and have conserved intron–exon structures. The resistant and susceptible haplotypes were highly conserved, with 98.8% overall nucleotide identity in a 39-kb region. Regions flanking these genes were also highly conserved between the two haplotypes, but differed between different sites within each haplotype. The *RB* and *rb* sequences exhibited 99.8% nucleotide identity, with only three synonymous mutations, one nonsynonymous mutation, and one 18-bp deletion. High nucleotide identity (>98.8%) was also observed between the other four pairs of homologs (Fig. 1). In



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1
MAEAFIQVLLDNLTSFLKGELVLLFGFQDEFQRLSSMFSTIQAVL
EDAQEKQLNNKPLENWLQKLNAATYEVDDILDEYKTKATRFSQSE
YGRYHPKVIPFRHKVGKRMDQVMKKLKAIAEERKNFHLHEKIVER
QAVRRETGSVLTEPQVYGRDKEKDEIVKILINNVSDAQHLSVLPI
LGMGGLGKTTLAQMVFNDQRVTEHFHSKIWICVSEDFDEKRLIKA
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EINKHSYTHMMSIGFAEVVFFYTLPP
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522
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FGDLPCLESLELHWG
SADVEYVEEVDIDVHSGFPT
RIRFPSLRELDIWDFGSLEGLLKREG
EEQFPVLEELIIHECPELTL
SSNLRALTSLRICYKVATSFPEEM
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LASLNALKSLKIQLCALESLPEEG
LEGLSSLTELFVEHNMLKCLPEG
LQHLTTLTSLKIRGFPQLIKRCEKGIGED
WHKISHIPNVNIYI
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Fig. 4. Structure of the *RB* gene and the deduced RB protein. (*A*) Physical structure of the *RB* gene. Two exons are indicated by open rectangles, and one intron is indicated by lines angled downward. (*B*) Predicted RB protein sequences. The potential leucine zipper motif and a heptad repeat motif are underlined. The three predicted kinase motifs of the NBS domain are shown in blue. Conserved motifs for plant resistance genes are shown in green. The two amino acid changes (E^{420}-K and K^{662}-M) caused by LR-PCR misincorporation are indicated in bold and underlined. The start point of the 3.6-kb deletion in *RGA2*-BAC is double underlined. The LRRs are aligned according to the consensus sequence LXXLXXLXXLXLXXN/CXXLXXLXX, where X represents any amino acid. The first L and the last two Ls are not highly conserved in different LRRs. Aliphatic residues L, I, M, V, and F are red; conversed N, C, and T residues are brown.

contrast, the paralogs exhibited only 79.6–85.6% nucleotide identity. No obvious sequence exchange between paralogs was found. Therefore, there is an obvious orthologous relationship between members located at the same position in resistant and susceptible haplotypes.

Diversifying selections have been detected in many different resistance gene systems (20). Selections on each site of the *RB* homologs were investigated by using model M2 in the program CODEML of PAML (12, 13). Eight sites were found to be under

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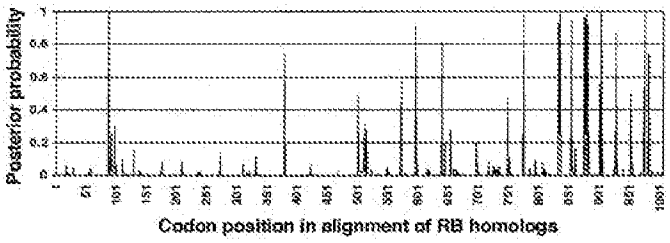


Fig. 5. The posterior probability of diversifying selection at each site in *RB* homologs. The *x* axis denotes codon position in the alignment of *RB* homologs. A site with posterior probability >0.95 is considered to be under significant diversifying selection.

diversifying selection, with posterior probability >0.95 (Fig. 5), seven of them located at the regions potentially encoding solvent-exposed residues within the LXXLXXLXLXXC/NXX motif of the LRR repeats, consistent with previous studies.

Discussion

We have demonstrated that transgenic Katahdin plants containing the *RB* gene show resistance to all tested isolates, including a "super race" that can overcome all 11 known R genes in potato. The *RB* gene encodes a polypeptide of 970 aa and belongs to the CC-NBS-LRR class of plant resistance genes (21). *RB* is more closely related to the I2 protein of tomato (30% identity, 47% similarity over 1,070 aa) than it is to any other known R protein (22). *RB* has limited similarity with the protein of *R1* (22% identity, 49% similarity over 902 aa), a gene derived from *S. demissum* that confers hypersensitive resistance to potato late blight (23). Many R genes are organized in clusters (24). Similarly, *RB* is a member of a four-gene family located within a 40-kb region on chromosome 8. *RB* transcript was detected in unchallenged plants, indicating that *RB* is expressed in the absence of the corresponding Avr-expressing pathogen, similar to other R genes that function in pathogen surveillance (25).

Sequence comparison between *RB* and its susceptible allele *rb* revealed a C^{1362} to G point mutation that creates a stop codon in the second exon at Tyr-454. Other than this stop codon within *rb*, the amino acid sequences deduced from *RB* and *rb* are highly similar, with only three synonymous point mutations (C^{28} to T, T^{2635} to C, and A^{2745} to G), a point mutation of T^{65} to C that changes valine to alanine, and a deletion of an 18-bp sequence that resulted in a loss of six amino acids (KIQLCC) in the 18th LRR repeat. It is likely that the premature stop codon in the *rb* sequence resulted in the loss of function, but the effects of other mutations, particularly the 18-bp deletion, remain unclear. *RGA2*-BAC, a chimera of *RB* and *RGA-tr* generated during BAC propagation, failed to complement *RB* function, suggesting that the last 151 aa in the LRR domain are essential for the function of *RB* resistance.

Both *RB* and *rb* contain 21 LRR repeats, whereas *RGA1*, *RGA3*, and *RGA4* contain 22 LRR repeats (Fig. 6, which is published as supporting information on the PNAS web site). The variation of LRR repeats may play a role in determining the recognition specificity of the RB protein. It has been demonstrated that expansion and contraction of LRR repeats are responsible for loss of function or recognition specificities of plant disease resistance genes. In flax, inactivation of the rust resistance gene *M* was associated with the loss of a single repeated unit within the LRR coding region (26). Sequence analysis of mutant *RPP5* alleles identified four duplicated LRR repeats in comparison to the wild-type *RPP5* gene (27). Recently, domain swapping and gene shuffling of tomato proteins Cf-4 and Cf-9 also demonstrated that variation in LRR copy number plays a major role in determining recognition specificity in these proteins (28).

R proteins may perceive the presence of more than one Avr proteins (29). Two recently identified R genes, RPW8.1 and RPW8.2, which consist of only a coiled-coil domain and a single N-terminal transmembrane domain, confer resistance to all tested isolates of four species of powdery mildew of *Arabidopsis*, indicating that RPW8-mediated resistance may not involve a gene-for-gene interaction (30). Dual recognition has been demonstrated in several cases. For example, *RPM1* recognizes two nonhomologous *P. syringae avr* genes (31). Similarly, the tomato *Mi* gene confers resistance both to the root-knot nematode and to potato aphid (32). The broad spectrum resistance against multiple races of *P. infestans* suggests that the RB protein may recognize conserved molecules from different races of the pathogen.

Gene *RB* shows an evolutionary pattern typical to Type II resistance genes (H.K., E. Nevo, and R. W. Michelmore, unpublished work). Gene *RB*, like the Type II *RGC2* resistance genes in lettuce, might be highly conserved in different genotypes or closely related species and present at high frequencies in natural populations. This is consistent with the observation that many accessions of *S. bulbocastanum* are highly resistant to all races of *P. infestans*. There might be purifying selection on *RB* orthologs, even at the hypervariable sites in the LRR motif, as observed in K orthologs in lettuce (H.K., E. Nevo, and R. W. Michelmore, unpublished work). This hypothesis can be tested when more *RB* orthologs become available.

Resistance of potato germplasm developed from *S. bulbocastanum* clone PT29 is effective against all known races of the late blight pathogen. There have been no reports that this resistance has been overcome by any of the *P. infestans* pathotypes. Transgenic Katahdin plants with the *RB* gene developed limited lesions on the lower leaves consistent with symptoms noted in field evaluations of the *RB*-containing progenies derived from *S. bulbocastanum* clone PT29 (5). The *RB* locus on chromosome 8 explains 62% of the genetic variation in late blight resistance in the progenies derived from *S. bulbocastanum* (8). Quantitative trait loci (QTL)-associated late blight resistances, which have been identified in various potato populations (33–35), may also contribute to the resistance of *S. bulbocastanum* clone PT29. Thus, *RB* may represent a substantial part, but not all, of the resistance contained within the genome of *S. bulbocastanum* clone PT29.

Currently, none of the major potato varieties grown in the United States contain resistance to US-8, the most prevalent genotype of *P. infestans*. Late blight-resistant germplasm has been developed from potato–*S. bulbocastanum* somatic hybrids (6). These materials can be valuable for breeding new late blight-resistant cultivars through marker-assisted selection. However, because the potato genome is tetraploid and highly heterogeneous, the production of late blight-resistant cultivars acceptable to industry may not be efficiently realized through repeated backcrosses of the *S. bulbocastanum*-derived germplasm to modern cultivars. Replacing old and disease-susceptible potato varieties has been an extremely slow process because these current varieties are well adapted to the processing industry. Russet Burbank, a late blight-susceptible variety released more than 100 years ago, still accounts for almost half of the potato acreage in the United States. With the deployment of the cloned *RB* gene it is now possible, through genetic engineering, to render the current popular potato varieties late blight-resistant.

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ORIGINAL PAPER

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Mapping of extreme resistance to PVY (Ry_{sto}) on chromosome XII using anther-culture-derived primary dihaploid potato lines

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Abstract The inheritance of extreme resistance to PVY (Ry_{sto}) by a single dominant locus was confirmed by obtaining a 1:1 segregation ratio in a virus inoculation test with 28 resistant (*Ryry*) to 29 susceptible (*ryry*) anther culture-derived dihaploid lines ($2n=2x=24$) from cv. "Assia" ($2n=4x=48$) having extreme resistance derived from *Solanum stoloniferum* in simplex constitution (*Ryryryry*). Twelve Ry_{sto} markers selected in AFLP assays using bulked segregant analysis were applied to 106 tested potato cultivars from Germany, The Netherlands and Poland and 19 potato cultivars were identified by these markers as extremely resistant to PVY in alignment with phenotypic data. The locus for extreme resistance (Ry_{sto}) to PVY was mapped on chromosome XII co-segregating with the SSR marker STM0003. The utility of anther-culture derived dihaploid potatoes for genetic marker development was demonstrated. Marker transferability from diploids to tetraploids provides an optimistic potential for marker-assisted selection in potato breeding programs.

Introduction

Potato virus Y (PVY) belongs to *Potyvirus*, the largest genus of plant viruses (Hooker 1981; Shukla et al. 1994), and is one of the most important viruses in *Solanaceae*. PVY infection can reduce potato production up to 80% (De Bokx and Huttinga 1981). Quick accumulation of virus concentration in tubers caused by the vegetative propagation requires an intensive seed production management. Breeding of resistant cultivars becomes more important, since a new strain, PVY^{NTN}, causes large necrotic ring blemishes on the tubers (Weidemann 1993; Le Romancer et al. 1994) and affects also the ware potato market, in contrast to the well-described PVY strains PVY^{O}, PVY^{N} and PVY^{C} (Jones 1990; Świeżyński 1994).

Different resistances to PVY are already known in potato. The extreme resistance mechanism is characterized by a strong reduction of virus replication in infected cells, whereas the hypersensitive resistance mechanism inhibits virus spread from cell to cell and through the vascular system (Ponz and Bruening 1986; Valkonen and Somersalo 1996; Bendahmane et al. 1999). For hypersensitivity, the Ny_{tbr} locus from *S. tuberosum* was mapped on chromosome IV (Celebi-Toprak et al. 2002). Extreme resistance to PVY was found in *S. tuberosum* ssp. *andigena* (Muñoz et al. 1975; Galvez and Brown 1980), *S. hougasii* (Cockerham 1970) and *Solanum stoloniferum* (Cockerham 1943). The extreme resistance from *S. stoloniferum* was defined as immunity and combines extreme resistance to virus Y and virus A (Cockerham 1943; Ross 1986). In most European cultivars, extreme resistance to PVY originates in *S. stoloniferum* owing to the introgression of the single dominant resistance gene Ry_{sto} into *S. tuberosum* by Ross (1952, 1958). Approximately 20 cultivars were listed as carrying the gene *Ry* from *S. stoloniferum* in Europe (Ross 1986). The locus Ry_{chc} from *S. chacoense* was mapped on chromosome IX (Hosaka et al. 2001). The resistance genes Ry_{adg} from *S. tuberosum* ssp. *andigena* (Hämäläinen et al. 1997) and Ry_{sto} from *S. stoloniferum* (Brigneti et al. 1997) were mapped on the same region of chromosome XI. However, marker utility was only given for Ry_{adg} (Hämäläinen 1999; Ruiz de Arcaute et al. 2002). Unreliable pedigree information for the population used by Brigneti

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et al. (1997) might be the reason (Gebhardt and Valkonen 2001; Flis et al. 2005).

Most genetic analysis in potato has been performed at diploid ($2n = 2x = 24$) level to reduce the complexity of the genome due to the highly heterozygous autotetraploid constitution of the cultivated potato ($2n = 4x = 48$). For ploidy reduction, parthenogenic (Hougas and Peloquin 1958; Hermsen and Verdenius 1973; van Breukelen 1975, 1977) and androgenic processes (Irikura and Sakaguchi 1972; Foroughi-Wehr et al. 1977; Jacobsen and Sopory 1978; Sopory et al. 1978; Wenzel and Uhrig 1981; Rokka et al. 1996) were used, whereby anther culture was only applicable for a few cultivars and breeding lines because of strong genotype specific influences of the donor plant on regeneration (Schwarzfischer et al. 2002). Primary dihaploid lines obtained by parthenogenesis were, for example, used for localization of the nematode resistance gene H1 on chromosome V (Pineda et al. 1993) and for QTL analysis (Douches and Freyre 1994).

In this study, anther-culture-derived dihaploid lines were used successfully for marker development in potato by combining AFLP analysis (Vos et al. 1995) and bulked segregant analysis (Michelmore et al. 1991). The applicability of the resulting markers for tagging Ry_{sto} at the tetraploid level was demonstrated, and marker-assisted breeding is now feasible.

Materials and methods

Plant material

A primary dihaploid ($2n = 2x = 24$) potato (*Solanum tuberosum* ssp. *tuberosum*) population with 57 individuals was used for virus resistance assay and for localization of the resistance locus. This mapping population was produced by anther culture from the German cultivar "Assia" ($2n = 4x = 48$) in 1996 and 1997 (Schwarzfischer et al. 2002). The cultivar "Assia" (*acl*, *adg*, *dms*, *sto*, *vrn*) from the cross MPI 65.346/19 and UP 5.332/10 was registered with extreme resistance to PVY, PVA and PVX by the Bundessortenamt (Beschreibende Sortenliste 1980). According to pedigree data, extreme resistance of cv. "Assia" was derived from *S. stoloniferum* (Ross 1986; Świeżyński et al. 1997). Detailed pedigree information is compiled in the potato pedigree database (http://www.dpw.wau.nl/pv). In addition, 10 resistant and susceptible potato cultivars ("Artis", "Bettina", "Forelle", "Kuras", "Laura", "Petra", "Sempra", "Sibu", "Tomba" and "Ute") were used for marker verification. Furthermore, 106 different potato cultivars from different European nations were analyzed for identification of cultivars with extreme resistance to PVY.

PVY inoculation

Before proceeding with the virus inoculation test, in vitro plant material of the mapping population was analyzed by ELISA (Enzyme-Linked Immunosorbent Assay) to exclude any prior PVY contamination. Each line was propagated in vitro. Twenty repeats were pricked out into 96-cell planting trays containing potting soil (Ø 3 cm) and hardened under a net in the greenhouse (13 h, 20°C day/11 h, 15°C night). Additionally, cultivars "Assia" and "Walli" were prepared as negative and positive controls. After 2 weeks, these lines were planted in pots (Ø 9 cm) in May 2000. Potato virus Y inoculation was performed after 3 weeks using mechanical inoculation methods with a mixture of four different strains (PVY^{o}, PVY^{c}, PVY^{N} and PVY^{NTN}). Two weeks after PVY inoculation, plants were observed for symptoms of primary infection and minitubers were harvested 4 months after inoculation. Tuber dormancy was artificially broken by treatment with Rindite and 20 tubers per line were planted separately in pots in the greenhouse on December 2000. When the plants had grown up to 15–20 cm 3 weeks after replanting, a few leaves of each pot were analyzed by ELISA (visual color reaction) and the plants were evaluated for visual symptoms. Six lines died before evaluation was completed. Resistant lines were defined as completely free of PVY in ELISA and without visible mosaic, wrinkle or necrotic symptoms in 20 repeats. Based on the results of the PVY-ELISA test and symptom evaluation, twelve selected resistant and susceptible lines with unequivocal test results in all repeats were composed in pools. Bulked segregant analysis was performed with pools of mixed DNA from eight lines and individual DNA from four lines.

DNA preparation

Genomic DNA was extracted from in vitro cultured plants according to CTAB methods (Saghai-Maroof et al. 1984) with modifications. Fresh plant material (6 g) was finely ground in a pre-chilled (−20°C) mortar using liquid nitrogen and transferred into a 50-ml Falcon tube containing 24 ml 2x-CTAB buffer. After vortex, the mixtures were incubated in a water bath at 60°C for 1 h. A 25-ml mixture of chloroform/isoamylalcohol (49:1) was added and the tube was inverted for 30 min at 60 rpm in an invert shaker (Reax2, Germany). After centrifugation at 5410 g for 15 min, the supernatants (24 ml) were transferred into new tubes containing 1 µl of RN*ase* (10 mg/l) and incubated at room temperature for at least 30 min or overnight. The chloroform extraction was repeated again for purification. DNA was precipitated by adding two-thirds volume of iso-propanol. The resulting DNA pellet was hooked with a glass rod and washed first with 0.2 M sodium acetate in 76% ethanol, then with 10 mM ammonium acetate in 76% ethanol. The DNA pellet was dried at room temperature and dissolved in TE buffer (pH 8.0). DNA concentration was adjusted to 500 ng/µl.

Molecular marker analysis

The non-radioactive AFLP analysis was performed with small modifications according to Hartl and Seefelder (1998) based on the "AFLP-Protocol for Public Release" version 2.0 from KeyGene Company, The Netherlands (Vos et al. 1995). Two restriction enzyme combinations were used to prepare template DNA: *Eco*RI/*Mse*I and *Pst*I/*Mse*I (New England Biolabs, England). Genomic DNA (250 ng) was first digested with 2.5 U of *Eco*RI or *Pst*I and 1.0 U of *Mse*I in 20-µl reaction volume at 37°C for 3 h. The adapters corresponding to each enzyme cleavage site were ligated to the restricted DNA at 37°C for 1 h. Preamplification of the restricted-ligated fragments was performed using primers complementary to the adapter sequence plus an 1-bp extension at their 3′end: *Eco*RI + A/*Mse*I + C and *Pst*I + G/*Mse*I + C. For selective amplification, the primers had the adapter sequence plus a 3-bp extension at the 3′end. *Eco*RI and *Pst*I primers were 5′end labeled with fluorescein. In total, 480 selective primer combinations were screened. The amplified DNA fragments were separated on pre-heated 5% polyacrylamide (Acrylamide:Bisacrylamide 19:1, Roth, Karlsruhe), 7 M Urea, 1X TBE denaturing sequence gel at constant power (50 W, 1,600 V and 250 mA) for 2 h. The amplified DNA fragments were visualized by a laser scanner (Typhoon 9200, Amersham-Pharmacia, Freiburg) under highly sensitive conditions (excitement wavelength: 488 nm, band-pass filter: 530 ± 15 nm) and documented with the software package: FRAGMENT ANALYSIS 1.1 (Amersham-Pharmacia, Freiburg). Polymorphic bands were scored by using the program AFLP-Quantar 1.0 (Keygene, The Netherlands). The published sequence characterized amplified region (SCAR) markers from Kasai et al. (2000) were used for marker verification in ten German potato cultivars. Simple sequenced repeats (SSRs) from Milbourne et al. (1998) were used for identifying linkage groups in the primary dihaploid population of 57 individuals to assign AFLP linkage groups to chromosomes: STM0001 (chromosome VI), STM0003 (XII), STM0004 (VII), STM0007 (XII), STM0010 (IX), STM0013 (V), STM0014 (VII, XII), STM0017 (IX), STM0019 (VI), STM0024 (VIII), STM0025 (XI), STM0028 (VII), STM0030 (XII), STM0031 (VII), STM0032 (XII), STM0038 (II), STM0051 (X), STM1021 (IX), STM1049 (I), STM1100 (VI), STM2005 (XI), STM2012 (X), STM3016 (IV). The amplified DNA fragments were separated under the same conditions as described above and stained with 1:10,000 diluted Vistra Green (Amersham Pharmacia, Freiburg) for 5 min. Bands were detected by laser scanner (Typhoon 9200).

The PCR marker GP 81 and CAPs markers GP 122 (*Eco*RV), GP 204 (*Taq*I) and GP 269 (*Dde*I) were tested in the dihaploid Assia population and in 8 resistant ("Assia", "Barbara", "Hinga", "Klepa", "Meduza", "Nimfy", "Pirola", "Ute") and 8 susceptible cultivars ("Amingo", "Christa", "Gloria", "Hansa", "Producent", "Sarturna", "Ukama", "Ulla") to compare the results with the mapped $Ry\text{-}f_{sto}$ gene of Flis et al. (2005).

Polymorphic bands from SSRs and AFLPs were tested for segregation distortion considering an X^2 test at a given significance level of $P = 0.05$ for one-to-one segregation. Linkage groups were constructed with one-to-one segregating markers, which were converted to F_2 backcross population mode under Kosambi mapping function using an LOD threshold of 5.0 and maximum distance of 25 cM in the Mapmaker program (Lander et al. 1987).

Results

Evaluation of PVY resistance

Potato virus Y was not detected in any in vitro material by ELISA before virus inoculation. The phenotypic response of susceptible lines was clearly visible mainly in the form of mosaic symptoms 2 weeks after mechanical inoculation of the seedlings in the greenhouse, however, many of the seedlings were too small to select the resistant lines precisely. The phenotypic symptoms caused by PVY were more clearly seen in plants grown from tubers (secondary infection) rather than in the infected seedlings. A phenotypic segregation of 23 resistant and 28 susceptible lines was obtained by PVY-ELISA and visual evaluation of symptoms. In the plants P 72 and P 76c, PVY was not detected by ELISA, but they showed some mild mosaic symptoms and weak growth (Table 1). The segregation of resistance showed a 1:1 ratio by an X^2 test at a significance level of $P = 0.05$. The genetic constitution of extreme resistance to PVY of "Assia" is inferred as simplex (*Ryryryry*) according to the segregation results.

Bulked segregant analysis

Out of 480 primer combinations, 27 AFLP marker candidates were identified by analysis of resistant (R) and susceptible (S) pools composed respectively of 12 rigorously selected resistant and susceptible lines. These marker candidates were tested again with individual DNA samples from each line of the R and S pool (Fig. 1). Finally, 15 Ry_{sto} AFLP markers from the 27 AFLP marker candidates were differentiated clearly as resistant and susceptible dihaploid lines in the bulked segregant analysis.

The 27 selected Ry_{sto} AFLP marker candidates from bulked segregant analysis were tested; in the six lines, which could not be analyzed in the inoculation test and in ten potato cultivars having reliable data for PVY-resistance as control for marker selection. Among the six lines, five showed the resistance-associated bands. Cultivars "Bettina", "Forelle" and "Ute" were registered to have extreme resistance to PVY (Beschreibende Sortenliste Kartoffeln 1979; 1982; 1988), "Kuras", "Sibu", "Petra" and "Tomba" were classified as highly resistant

Table 1 Results of the potato virus Y inoculation test for 51 dihaploid lines

Line	ELISA[a]	Phenotypic evaluation[b]	Pool[c]		Line	ELISA[a]	Phenotypic evaluation[b]	Pool[c]	
			R	S				R	S
P 1	+	S		X	P 69	–	R	X	
P 2	+	S			P 72	–	S		
P 3	+	S			P 81	–	R	X	
P 3.1	+	S			P 85	+	S		X
P 4	+	S			P 86	–	R	X	
P 5	–	R			P 87	+	S		X
P 9.1.1	+	S		X	P 98	–	R	X	
P 11	–	R			P 99	–	R		
P 12.2	+	S		X	P 102	–	R	X	
P 15	–	R	X		P 103	+	S		X
P 19	+	S		X	P 104	+	S		
P 26	+	S			P 109	+	S		
P 27	–	R	X		P 110	–	R		
P 30	+	S			P 113	–	R		
P 33	–	R			P 115	–	R		
P 34	–	R			P 118	+	S		
P 35	+	S		X	P 120	–	R	X	
P 36	+	S			P 123	+	S		
P 39	–	R	X		P 125	–	R		
P 41	–	R	X		P 63a	–	R	X	
P 49	–	R	X		P 76a	+	S		X
P 50	+	S			P 76b	+	S		X
P 51	+	S			P 76c	–	S		
P 60	+	S		X	P 76d	–	R		
P 61	–	R			P 78a	+	S		X
P 66	+	S							

[a]Reaction to PVY by ELISA: "-" no reaction (resistant), "+" positive reaction (susceptible)
[b]R stands for resistance and S for susceptibility

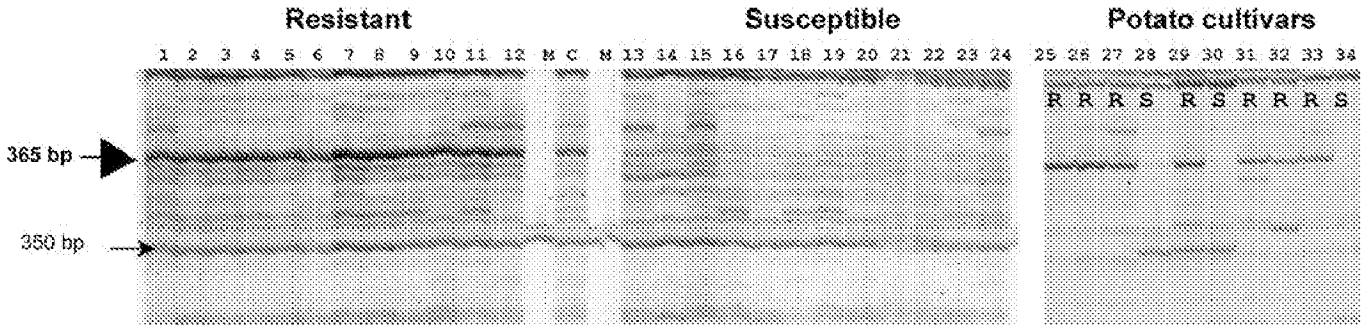


Fig. 1 Ry_{sto} AFLP marker (E+ACC/M+CTC-365) in dihaploid lines and ten potato cultivars. *Lanes 1–12* resistant dihaploid lines, *lanes 13–24* susceptible dihaploid lines, *lanes 25–34* potato cultivars ("Bettina", "Forelle", "Kuras", "Laura", "Petra", "Artis", "Sibu", "Tomba", "Ute" and "Sempra"), *M* 50–500 bp size marker, *C* "Assia" (control)

to potato virus Y while "Artis" and "Sempra" were recorded with low resistance to PVY (Beschreibende Sortenliste 2001). Cultivar "Laura" is recorded as highly resistant, whereas to our experience the resistance level is medium. The Ry_{sto} AFLP markers selected in ten potato varieties agreed with the selected markers in dihaploid lines from bulked segregant analysis (Fig. 1) except marker E+ACT/M+CGA-163.

By contrast, when the SCAR markers published by Kasai et al. (2000) were tested in these ten potato cultivars, no resistant cultivar was detected. Simultaneously the CAPs marker GP 122 (*Eco*RV) was tested, however, no polymorphism was observed.

Mapping of Ry_{sto} with AFLP and SSR markers

One locus (111 bp), amplified by the SSR primer STM0003, was detected as a Ry_{sto} marker by co-segregating with Ry_{sto} AFLP markers. For linkage analysis, 306 one-to-one segregating AFLP markers were selected from, in total, 620 polymorphic bands produced by 27 marker candidates and 7 additional AFLP primer combinations. The Ry_{sto} markers were tightly linked with LOD grouping thresholds over 3.0. According to linkage analysis, the Ry_{sto} resistance markers are linked to chromosome XII (Fig. 2) based on the reference maps from Milbourne et al. (1998) and Gebhardt et al. (1991).

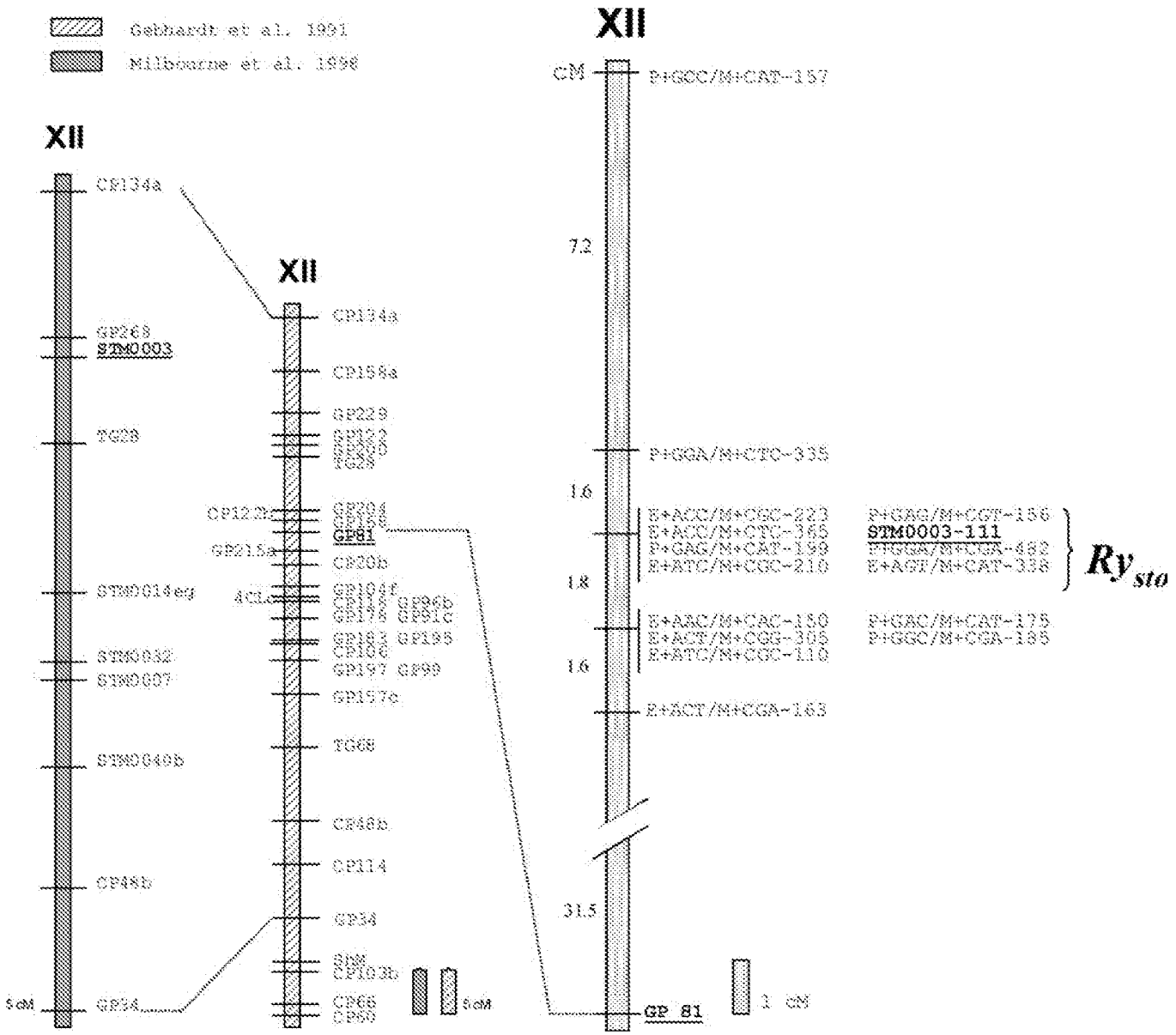


Fig. 2 Potato linkage group XII with the Ry_{sto} locus for extreme resistance to PVY. Map distances are given in centimorgans (cM)

In addition, the marker GP 81 was linked with a map distance of 34.9 cM. If one line (P3) was removed in our linkage analysis, five more markers (E+AAC/M+CAC-150, E+ACT/M+CGG-305, E+ATC/M+CGC-110, P+GAC/M+CAT-175, P+GGC/M+CGA-185) with a distance of 1.8 cM also co-segregated with STM0003-111. So, P3 was detected as a recombinant plant.

Identification of potato cultivars with extreme resistance to PVY

Selected Ry_{sto} markers were applied to 106 potato cultivars (Table 2). Two different AFLP marker types (A, B) could be distinguished according to their cultivar profile (Fig. 3). Nineteen cultivars were detected as extremely resistant to PVY by observing nine AFLP markers from Type A (P+GAC/M+CAT-175, P+GAG/M+CAT-199, P+GAG/M+CGT-156, P+GGA/M+CGA-482, P+GGA/M+CTC-335, P+GGC/M+CGA-185, E+AAC/M+CAC-150, E+ACC/M+CTC-365, E+AGT/M+CAT-338) and the SSR marker STM0003-111 in agreement with phenotypic reports (Table 2): "Arosa", "Alwara", "Assia", "Bettina", "Dania"(=Rania), "Forelle", "Franzi" (1977), "Hinga", "Jumbo", "Klepa", "Kuras", "Meduza", "Nimfy", "Oktan", "Petra", "Sibu", "Solara", "Tomba" and "Ute". Marker type B (E+ACC/M+CGC-223, E+ACT/M+CGG-305, E+ATC/M+CGC-110) did not detect "Alwara", "Arosa", "Hinga", "Meduza" and "Nimfy" out of the 19 selected

Table 2 Analysis of 106 potato varieties with Ry_{sto}markers

Cultivar	Resistance level to PVY[a]			Presence of the Ry_{sto} marker[d]			Cultivar	Resistance level to PVY[a]			Presence of the Ry_{sto} marker[d]		
				AFLP		SSR					AFLP		SSR
	D[b]	NL[b]	Po[b]	A	B	$STM0003_{111}$		D[b]	NL[b]	Po[b]	A	B	$STM0003_{111}$
Acapella	2			−	−	−	Klepa			E.R.[c]	+	+	+
Adora		6.5		−	−	−	Kuras		9.5		+	+	+
Agria	2			−	−	−	Laura	1			−	−	−
Alwara	1			+	−	+	Likaria	5			−	−	−
Amigo	7			−	−	−	Liseta		9.5		−	−	−
Angela	5			−	−	−	Marabel	1			−	−	−
Arosa	1			+	−	+	Marella	1			−	−	−
Artana	6			−	−	−	Maxilla	2			−	−	−
Artis	4			−	−	−	Meduza			E.R.[c]	+	−	+
Assia	1*			+	+	+	Miriam	3			−	−	−
Astarte		8.5		−	−	−	Mondial		7.5		−	−	−
Astoria	2			−	−	−	Moni	2			−	−	−
Atica	3			−	−	−	Monza	1			−	−	−
Baronesse	2			−	−	−	Natalie	2			−	−	−
Bettina	1*			+	+	+	Nimfy			E.R.[c]	+/−	−	+
Brigit	2			−	−	−	Nora	1			−	−	−
Bolero	1			−	−	−	Oktan	1			+	+	+
Bonanza	1			−	−	−	Pallina	4			−	−	−
Calla	2			−	−	−	Panda	3			−	−	−
Camilla	1			−	−	−	Patrona	5			−	−	−
Carmona	2			−	−	−	Petra	1			+	+	+
Christa	6			−	−	−	Ponto	3			−	−	−
Colette	1			−	−	−	Power	3			−	−	−
Combi	1			−	−	−	Presto	1			−	−	−
Dania (=Rania)	1			+	+	+	Producent	6			−	−	−
Debora	4			−	−	−	Quarta	4			−	−	−
Desiree	2			−	−	−	Rebecca	2			−	−	−
Ditta	2			−	−	−	Renate	3			−	−	−
Elkana		7.5		−	−	−	Rikea	2			−	−	−
Exempla	3			−	−	−	Rita	2			−	−	−
Exquisa	2			−	−	−	Rincona	2			−	−	−
Fausta	3			−	−	−	Salome	1			−	−	−
Festien	1			−	−	−	Satina	1			−	−	−
Flora	2			−	−	−	Saturna	6			−	−	−
Forelle	1*			+	+	+	Scala	2			−	−	−
Fox	1			−	−	−	Selma	3			−	−	−
Franca	3			−	−	−	Sempra	2			−	−	−
Franzi	1*			+	+	+	Seresta		7.0		−	−	−
Franzi	2			−	−	−	Sibu	1			+	+	+
Frieda	3			−	−	−	Sieglinde	3			−	−	−
Gloria	6			−	−	−	Sjamero	1			−	−	−
Granola	3			−	−	−	Solara	1			+	+	+
Gunda	5			−	−	−	Sommergold	1			−	−	−
Hansa	8			−	−	−	Spunta		7.5		−	−	−
Hinga			E.R.[c]	+	−	+	Terra	1			−	−	−
Indira	2			−	−	−	Tessi	2			−	−	−
Impala	4			−	−	−	Tomba	1			+	+	+
Irmgard	4			−	−	−	Ukama	6			−	−	−
Jetta	4			−	−	−	Ulla	8			−	−	−
Juliane	2			−	−	−	Ulme	1			−	−	−
Jumbo	1*			+	+	+	Ute	1*			+	+	+
Kardent		7.5		−	−	−	Velox	4			−	−	−
Kartel		4.5		−	−	−	Walli	3			−	−	−

1*: Registered extremely resistant cultivar to potato virus Y by Bundessortenamt

[a]Resistance level to PVY: one (highly resistant) to nine (highly susceptible) in Germany, nine (highly resistant) to one (highly susceptible) in The Netherlands

[b]Nation: *D* Germany, *NL* The Netherlands, *Po* Poland

E.R.[c] Extremely resistant cultivar in Poland referenced by Flis et al. (2005)

[d]Ry_{sto} AFLP and SSR markers: + = present, − = absent, +/− = unclear to score, A and B indicate two different Ry_{sto} AFLP marker types

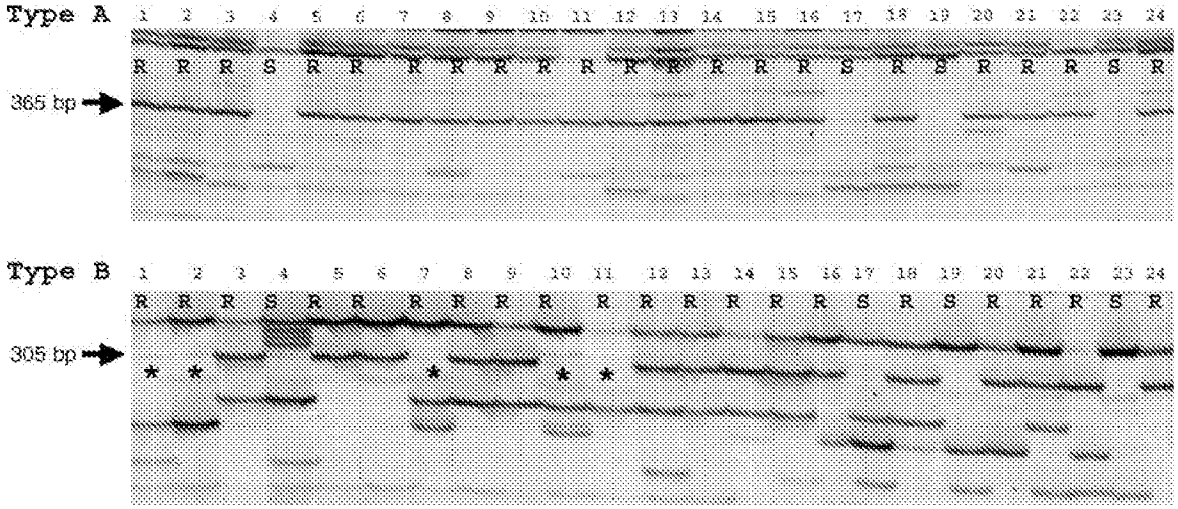


Fig. 3 Ry_{sto} AFLP marker types in 24 cultivated potatoes having a high level of PVY resistance. Type A: Ry_{sto}AFLP marker(-E+ACC/M+CTC-365), Type B: Ry_{sto}AFLP marker (E+ACT/M+CGG-305). *Lane 1* "Alwara", *lane 2* "Arosa", *lane 3* "Dania"(=Rania), *lane 4* "Fox", *lane 5, 6* "Franzi", *lane 7* "Hinga", *lane 8* "Jumbo", *lane 9* "Klepa", *lane 10* "Meduza", *lane 11* "Nimfy", *lane 12* "Oktan", *lane 13* "Solara", *lane 14* "Bettina", *lane 15* "Forelle", *lane 16* "Kuras", *lane 17* "Laura", *lane 18* "Petra", *lane 19* "Artis", *lane 20* "Sibu", *lane 21* "Tomba", *lane 22* "Ute", *lane 23* "Sempra" and *lane 24* "Assia", *Asterisk* (*) indicates that these cultivars were not detected by marker type B

cultivars (Fig 3, Table 2). Three markers (P+GCC/M+CAT-157, E+ACT/M+CGA-163, E+ATC/M+CGC-210) with a far distance (8.8 cM, 3.4 cM and 1.8 cM) to Ry_{sto}, were not selectable in 106 potato varieties. From two markers (E+ATC/M+CGC-210, E+ATC/M+CGC-110) derived from the same selective primer combination and with a map distance of 1.8 cM, just the marker E+ATC/M+CGC-110 identified the extreme resistant potato cultivars. The Polish cultivar "Nimfy" showed additional differences in marker pattern because the target band was unclear with the marker P+GAC/M+CAT-175 and the band of marker P+GGA/M+CGA-482 was absent.

Discussion

In this work, molecular markers for potato were established by the use of anther culture-derived plants. Anther culture of potatoes is difficult and there are some limitations in obtaining a large population because of strong genotype specific influences of the donor plant on regeneration (Jacobsen and Sopory 1978; Wenzel and Uhrig 1981; Uhrig 1985; Rokka et al. 1996; Schwarzfischer et al. 2002). Nevertheless, this unusual mapping strategy was very successful for marker development. Recently, anther-culture-derived monoploids have also been the basic material for linkage analysis (Tai et al. 2000; Varrieur 2002).

Our population size with 57 individuals was relatively small for localizing the Ry_{sto} locus. However, often small diploid populations with a size of 67 individuals (Gebhardt et al. 1991) or 68 individuals (Van Eck et al. 1995) were used for map construction. Furthermore, Hämäläinen et al. (1997) also succeeded in localizing the Ry_{adg} locus on chromosome XI with a dihaploid population of 54 individuals.

The male sterile cultivar "Assia" was the only one in our laboratory which showed good regeneration results in anther culture. Furthermore, also a few lines could be regenerated from the cultivars "Petra" and "Ute", which are also male sterile. Dihaploids from the cultivar "Petra" were also induced by Rokka et al. (1996). In conclusion, male sterility does not influence the androgeneric process.

All examined cultivars with known extreme resistance to PVY were detected by our markers. The resistance level of Polish cultivars "Hinga", Klepa", "Meduza" and "Nimfy" was kindly confirmed by Dr. Zimnoch-Guzowska at the Plant Breeding and Acclimatization Institute (IHAR) in Poland and was recently reported by Flis et al. (2005). Further cultivars were selected which were registered with very high resistance to PVY but not with extreme resistance, for example, "Alwara", "Arosa", "Dania"(="Rania"), "Oktan", "Petra", "Solara" and "Tomba". These cultivars presumably had never been tested for extreme resistance to PVY because this trait will only be examined by the Bundessortenamt by breeders' request. The cultivar "Fox" has a high resistance level to PVY and is derived from the same cross parents as "Assia". However, the loss of extreme resistance was confirmed by the graft inoculation test at the breeding station Uniplanta-Saatzucht KG (personal communication).

Previous studies on mapping of PVY resistance genes (Ry_{sto} and Ry_{adg}) revealed the region between the probes GP 125 and CT 182 on chromosome XI (Brigneti et al. 1997; Hämäläinen et al. 1997; Solomon-Blackburn and Barker 2001). In agreement with the results of Ruiz de Arcaute et al. (2002), we could not apply the Ry_{adg}

SCAR markers (Sorri et al. 1999; Kasai et al. 2000) for selection of PVY resistant cultivars in the spectrum of German potato cultivars and we also failed to locate the Ry_{sto} gene locus on chromosome XI. In contradiction with the earlier hypothesis of Brigneti et al. (1997), Ry_{adg} and Ry_{sto} represent different genes on different chromosomes. Therefore, marker utility must be considered dependent on the genetic background of the test material.

Our results indicate a localization of Ry_{sto} on chromosome XII according to the co-segregation with a locus amplified by the STM0003 primer, mapped to the region between the probes GP268 and TG28 on chromosome XII by Milbourne et al. (1998). They obtained a PCR product with 141 bp by STM0003, whereas in our population, an additional band (111 bp) was amplified. Interestingly, this band co-segregates with all resistant lines in the dihaploid Assia population and was only observed in the extreme resistant potato cultivars derived from *S. stoloniferum*. Additionally, the probe GP 81 confirmed the Ry_{sto} localization on chromosome XII based on the reference map of Gebhardt et al. (1991).

Recently, Flis et al. (2005) mapped the locus *Ry-f*$_{sto}$ on the chromosome XII. In comparison, the closely linked marker GP 122_{718} did not deliver polymorphism in our dihaploid Assia population. In contrast to Flis et al. (2005), we also failed to select the extremely resistant cultivars "Assia", "Barbara", "Hinga", "Klepa", "Meduza", "Nimfy", "Pirola" and "Ute". Only marker GP 81 could be linked to our Ry_{sto} with a map distance of 34.9 cM. This result indicates that the loci *Ry-f*$_{sto}$ and Ry_{sto} could represent two different genes for extreme resistance to PVY, both situated on chromosome XII.

There were two different Ry_{sto}AFLP marker types (A, B) according to their response to the cultivars "Alwara", "Arosa", "Meduza", "Hinga" and "Nimfy". We argue that recombination events occurred in this marker-specific region in earlier pedigrees. The polish cultivar "Nimfy" showed in addition differences in reaction profiles to AFLP marker type A, this maybe caused by methylation. The used restriction enzyme *Pst*I is reported to be much more sensitive to cytosine methylation than *Eco*RI and *Sac*I according to segregation data from a heterozygous diploid potato population in AFLPs (Isidore et al. 2003).

As extreme resistance to PVY (Ry_{sto}) is inherited by one single dominant gene, marker transfer from diploid to tetraploid level was possible. With our results based on good phenotypic evaluation data, exact pedigree information and well-established molecular methods, we can start a very precise resistance breeding strategy based on marker-assisted selection for the important breeding trait extreme resistance to PVY (Ry_{sto}). Ry_{sto} AFLP markers (Type A) provide reliable information to be converted to Site Target Sequence (STS) markers. Therefore selection of PVY resistant genotypes could be done very exactly in early stages of the breeding process, for example, in the seedling stage with small leaf samples by application of simple analytical methods independent of environmental influences. The additional costs for MAS and man power will be saved by reduction of greenhouse and field area and by minimization of laborious resistance tests for several years.

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Development of STS Markers for Selection of Extreme Resistance (Ry_{sto}) to PVY and Maternal Pedigree Analysis of Extremely Resistant Cultivars

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Abstract Two Ry_{sto} STS markers, YES3-3A (341 bp) and YES3-3B (286 bp), were successfully developed from the Ry_{sto} AFLP marker E+ACC/M+CTC-365. Both STS markers identified all 38 extremely resistant potato cultivars out of 188 European potato varieties from Germany, Hungary, Poland and The Netherlands, and also identified four extremely resistant MPI breeding lines out of five tested lines, in agreement with the phenotypic data of resistance level to PVY. In parallel, three different plastid-mitochondrial configurations (W/α, W/γ, and T/β) were analyzed by the PCR markers ALC_1,3 and ALM_4,5. Thirty-eight potato varieties were classified as the W/α cytoplasm type, 51 potato varieties were identified as carrying the W/γ cytoplasm type, and 99 potato varieties analyzed had the T/β cytoplasm type. All 38 marker-selected extremely resistant potato cultivars and four extremely resistant MPI breeding lines were identified as having the mitochondrial γ type in association with male sterility. Male sterility was validated by pollen germination tests in 38 potato cultivars carrying the mitochondrial α type or γ type. The male sterility of 17 extremely resistant potato cultivars and five potato cultivars carrying the W/γ cytoplasm type was confirmed by obtaining scarcely any or only few or wrinkled pollen grains and no subsequent pollen germination. On the other hand, pollen germination in all 16 potato cultivars with the W/α cytoplasm type was observed with different efficiency due to different genotypes. The Ry_{sto} gene of extremely resistant potato cultivars and MPI breeding lines traced back to *Solanum stoloniferum*, and the maternal pedigree analysis of these cultivars clustered into three groups depending on three different primary crosses with *S. stoloniferum*-hybrids. This pedigree analysis confirmed the precision of Ry_{sto} STS marker selection tagging the maternally inherited Ry_{sto} gene derived from MPI breeding lines.

Keywords Male sterility · Mitochondrial γ type · Marker assisted selection (MAS) · *Solanum stoloniferum* · *Solanum tuberosum*

Abbreviations

SSR	simple sequence repeat
STS	sequence tagged site
PVY	potato virus Y
AFLP	amplified fragment length polymorphism

Introduction

Potato virus Y (PVY), belonging to *Potyvirus*, along with potato virus A (PVA), is one of the most important viruses in *Solanaceae* (tomato, tobacco, pepper). PVY is the major viral potato pathogen, and reduces crop yield up to 80% (De Bokx and Huttinga 1981). PVY is more common than the Potato Leaf Roll Virus (PLRV) in some parts of Europe. There are three main PVY strain groups: PVY^O, PVY^N and PVY^C (Jones 1990; Świeżyński 1994). A new strain, PVY^{NTN}, which causes large necrotic ring blemishes on the tubers, affects the ware potato market (Weidemann 1993; Le Romancer et al. 1994). Vegetative propagated crops require intensive seed production management; however, a more effective strategy for avoiding viral

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disease in potatoes is breeding for resistance to viral infections, regardless of the appearance of new strains.

There are two major types of single dominantly-inherited resistance genes to PVY in cultivated and wild potato species (*Solanum* spp.); namely, hypersensitive response (*Ny*) and extreme resistance (*Ry*). The cell death response, known as the hypersensitive response mechanism, inhibits viral spread from cell-to-cell and through the vascular system, whereas the extreme resistance mechanism is characterized by a strong reduction of virus replication in infected cells (Ponz and Bruening 1986; Valkonen and Somersalo 1996; Solomon-Blackburn and Barker 2001).

Focusing on extreme resistance to PVY, there are three known resistance genes (Ry_{adg}, Ry_{chc} and Ry_{sto}) found in *Solanum tuberosum* ssp. *andigena*, *Solanum chacoense*, *Solanum hougasii* and *Solanum stoloniferum* (Muñoz et al. 1975, Galvez and Brown 1980; Cockerham 1943, 1970). These three *Ry*-genes, which are single dominant genes that provide complete protection, have been intensively studied and applied in practical potato breeding programs. Mendoza et al. (1996) reported that the International Potato Center (CIP) leads a project to produce breeding lines with triplex or quadruplex immunity (Ry_{adg}). The resistance gene Ry_{adg} from *S. tuberosum* ssp. *andigena* was localized on chromosome XI (Hämäläinen et al. 1997), in the same region as the resistance gene (Na_{adg}), which induces a hypersensitive response to PVA (Hämäläinen et al. 1998, 2000). Subsequently, the Ry_{adg} SCAR marker was developed by Kasai et al. (2000) and is currently utilized in a potato breeding program (Ruiz de Arcaute et al. 2002). The gene Ry_{chc} from *S. chacoense* was mapped onto chromosome IX with a RAPD marker tightly linked to the Ry_{chc} locus (Hosaka et al. 2001; Sato et al. 2006). This marker was combined with a 1-min DNA extraction method applied in breeding practice (Hosaka 2004).

One further gene source is the Ry_{sto} from *S. stoloniferum* Schlechtd., which is a tetraploid ($2n=4x=48$) Mexican wild potato species that belongs to *Longipedicellata* Buk. and is very polymorphic, with locally abundant forms differing in the degrees of leaf dissection and flower color (from white to purple) (Cockerham 1970; Hawkes 1992). The Ry_{sto} gene was first reported by Cockerham (1943). The Ry_{sto}-mediated resistance gene is reported to confer resistance against all strains of PVY. In addition, some genotypes carrying Ry_{sto} also react with extreme resistance to PVA, a virus related to PVY, while others do not (Ross 1952, 1986; Cockerham 1970; Barker 1997). The various accessions of Mexican *S. stoloniferum* were reported in Cockerham (1970), who defined a comprehensive extreme resistance as one of seven phenotypic responses by evaluating the combined resistance reactions to PVY and PVA in populations derived from various accessions of *S. stoloniferum*. Since extreme resistance to PVY has been introgressed into *Solanum tuberosum* in three to seven backcross generations by Ross (1952, 1958), most European extremely resistant potato cultivars carry this resistance gene via MPI breeding lines derived from *S. stoloniferum* (Ross 1986). The cultivars and breeding lines carrying the Ry_{sto} gene are known to show male sterility (Ross 1986), and the Ry_{sto} gene has been inherited from the female parent in the classical potato breeding program. Cytoplasmic male sterility (CMS) is inherited from the maternal parent through the mitochondrial genome. By identifying different plastid-mitochondria configurations with molecular markers, Lössl et al. (1999, 2000) reported that the plastid-mitochondrial W/γ type is associated with the phenotype of cytoplasmic male sterility in potato.

The Ry_{sto} and the $Ry\text{-}f_{sto}$ loci were recently located on chromosome XII (Song et al. 2005; Flis et al. 2005), however, a practical and accurate selection marker for potato breeding has not been feasible until now, even though the Ry_{sto} gene has such importance. This study presents the successful development of new Ry_{sto} STS markers from one Ry_{sto} AFLP marker, and also demonstrates the precision of these markers by identifying extremely resistant potato varieties and lines. The data were confirmed by maternal pedigree analysis of the Ry_{sto} gene and cytoplasm marker analysis, as well as a pollen germination test with European potato varieties.

Materials and Methods

Plant Material

The 188 tested potato cultivars from Germany, Hungary, Poland and The Netherlands were collected at the Bavarian State Research Center for Agriculture (LfL), Institute for Crop Science and Plant Breeding in Freising, Germany. The cultivars Alpha, Barbara, Cordia, Corine, Darwina, Eigenheimer, Fina, Frisia, Furore, Gamma, Jaerla, Mara, Pino, Pirola, Reinhort, Roeslau, Sira, Sirtema, Sissi, and Tanja were provided from the German gene bank, Institute of Plant Genetics and Crop Plant Research in Groß Lüsewitz, as along with five MPI breeding lines: MPI 44.1016/10, MPI 50.140/5, MPI 54.4129/288, MPI 55.957/54 and MPI 64.956/68. Two Hungarian potato cultivars (Magyar Rozsa and Szignal) were supplied from the Plant Breeding and Acclimatization Institute (IHAR) in Radzików, Poland. Dr. J. Taller at the University of Veszprem, Hungary, provided the cultivar 'White Lady' and confirmed the resistance levels of these Hungarian potato cultivars. The resistance levels of German potato cultivars were found on the National listing of potato varieties *Beschreibende Sortenliste Kartoffeln* (1979, 1983, 1988, 1993, 1996, 2001, 2003, 2004, 2005 and 2006) by the *Bundessortenamt*, and the resistance levels of Dutch, Hungarian and Polish potato cultivars were found on the

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European cultivated potato database of the Scottish Agricultural Science Agency (SASA) (http://www.europotato.org/index.htm).

Preparation of Genomic DNA

DNA extraction was performed according to CTAB methods (Saghai-Maroof et al. 1984) with modifications (Song et al. 2005). Freshly harvested *in vitro* cultured plants or young leaves of field grown plants (250 mg) were finely homogenized with 1 ml of 2×-CTAB buffer containing freshly mixed 2% (*v*/*v*) 2-mercaptoethanol.

Cloning of the Ry_{sto} AFLP Marker Fragment

The non-radioactive AFLP procedure was performed as detailed in Song et al. (2005). The Ry_{sto} AFLP marker E+ACC/M+CTC-365 from the Assia cultivar (Song et al. 2005) was excised from a 5% polyacrylamide gel. The target fragment was re-hydrated in a tube containing 100 µl of sterile water at room temperature for 2 min. After vortexing, the suspension was centrifuged at 4,000×*g* for 1 min, and 50 µl of the supernatant was transferred into a new tube. One microliter of supernatant was used in a 20 µl PCR reaction using the same pre-selective primers (E01 5′-GACTGCGTACCAATTCA-3′/M02 5′-GATGAGTCCT GAGTAAC-3′) under the same PCR profile as the second amplification in AFLP analysis. The amplified DNA fragment of the Ry_{sto} AFLP marker E+ACC/M+CTC-365 was separated using a 2% agarose gel containing ethidium bromide in 0.5×-TBE buffer. After electrophoresis, the target fragment was eluted by purifying with a JetSorb extraction kit (GENOMED, Germany) and cloned into a pCR®4-TOPO® vector using the TOPO TA® cloning kit (Invitrogen, Germany) according to the instruction manual. The cloned plasmids were isolated with a boiling preparation (Sambrook et al. 1989). The insertion of the DNA fragment into the plasmids was confirmed on a 1.8% agarose gel after the restriction enzyme reaction with *Pst*I. Plasmid DNA was purified by the Jetquick Plasmid Miniprep Spin kit (GENOMED) and sequenced by the company MWG (Munich, Germany). The sequence data were analyzed with the sequence analysis program Vector NTI™ version 6.0 (Invitrogen, USA).

Development of STS (Sequence Tagged Site) Markers

Primers were designed in alignment with sequences by taking into consideration a GC content over 40% and an annealing temperature of 50°C to 60°C. Designed primers were selected in each of seven extremely resistant and seven susceptible cultivars. The selected STS markers were applied to 188 potato varieties and MPI breeding lines.

Plastid-mitochondrial Configuration and Pollen Germination Test

The different plastid (W, T) and mitochondrial (α, β, γ) types of cultivated potatoes were analyzed with the specific primers ALC_1/3 (5′-TAGAATCAGGAGGTCTT-3′/5′-TTACTCACGGCAATC-3′) and ALM_4/5 (5′-AATAATCTTCCAAGCGGAGAG-3′/5′-AAGACTCGT GATTCAGGCAAT-3′) according to Lössl et al. (2000). In comparison, a pollen germination test was performed with 38 cultivated potatoes carrying the mitochondrial α type or γ type, both are coupled with W plastid in cultivated potatoes (Lössl et al. 1999, 2000). The harvested flowers from the field were dried on a paper plate at room temperature overnight. The gathered pollens were incubated in a Petri-dish (30 mm diameter) containing 2 ml pollen germination solution (100 mg L^{-1} boric acid, 300 mg L^{-1} calcium nitrate, 200 mg L^{-1} magnesium sulphate, 100 mg L^{-1} potassium nitrate, 15% (*w*/*v*) sucrose, pH 5.8, autoclaved) according to Schweis (1992). After 2 h, pollen germination was observed under a light microscope with a total magnification of ×200 or ×400 (Axioskop, Zeiss, Germany).

Pedigree Analysis

The pedigree information of the cultivars selected by the Ry_{sto} marker was compiled from Świeżyński et al. (1997) and the Potato Pedigree Database from Wageningen, The Netherlands (http://www.dpw.wau.nl/pv/index.htm). For some Polish cultivars, pedigree data were received from IHAR. The data for the Tomba and Oktan cultivars were kindly provided by Dr. Hofferbert from the Böhm-Nordkartoffel breeding station, Epsdorf, Germany. The pedigrees of the Jumbo, Maxi, Assia, Arosa and Alwara cultivars were received from the potato breeding department of the LfL.

Results

Ry_{sto} STS Marker Test in Cultivated Potatoes

Two different sequences of 342 bp (BV725479) and 341 bp (BV725480) were obtained from the cloned fragment of the Ry_{sto} AFLP marker E+ACC/M+CTC-365. Based on these sequence data, three primer pairs were designed and applied to selected extremely resistant and susceptible potato cultivars under the optimized PCR conditions summarized in Table 1. The primer pair 1F/1R amplified the same DNA fragment (269 bp) in both resistant and susceptible cultivars, whereas both STS markers, YES3-3A and YES3-3B, identified the extremely resistant potatoes by presenting different size fragments of 341 and 284 bp (Fig. 1) that were amplified by the same forward primer (3F) and two different

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Table 1 Designed STS primers and PCR cycle conditions

Marker/primer	Sequence (5′→3′)	Size (bp)	PCR cycles
YES3-1			
1F	CCACTAGATTGGAATCATACC	269	94°C 2 min 10 cycles (94°C 40 s, 56°C 40 s, 72°C 60 s)
1R	GACCCAAATTCTCTCCAAAT		30 cycles (94°C 40 s, 53°C 40 s, 72°C 60 s) 72°C 5 min
YES3-3A			
3F	TAACTCAAGCGGAATAACCC	341	94°C 2 min 10 cycles (94°C 40 s, 60°C 40 s, 72°C 60 s)
3A	AATTCACCTGTTTACATGCTTCTTGTG		30 cycles (94°C 40 s, 55°C 40 s, 72°C 60 s) 72°C 5 min
YES3-3B			
3F	TAACTCAAGCGGAATAACCC	284	94°C 2 min 10 cycles (94°C 40 s, 55°C 40 s, 72°C 60 s)
3B	CATGAGATTGCCTTTGGTTA		30 cycles (94°C 40 s, 53°C 40 s, 72°C 60 s) 72°C 5 min

reverse primers (3A and 3B). Depending on the genotype, any PCR products was amplified by the YES-3A marker, for example, lane 16 and 17 in Fig. 1. The YES3-3B marker required a high percentage agarose gel (over 2%) for identification of the target fragment in comparison to the YES3-3A marker (1.2%). Both STS markers were applied to five MPI breeding lines and 188 potato varieties collected from Germany, Hungary, Poland and The Netherlands.

The STS markers YES3-3A and YES3-3B identified 38 potato cultivars (Alwara, Amado, Arosa, Assia, Barbara, Bettina, Bison, Bzura, Cordia, Esta, Fanal, Forelle, Franzi, Heidrun, Hinga, Jaqueline, Jumbo, Klepa, Kuba, Kuras,

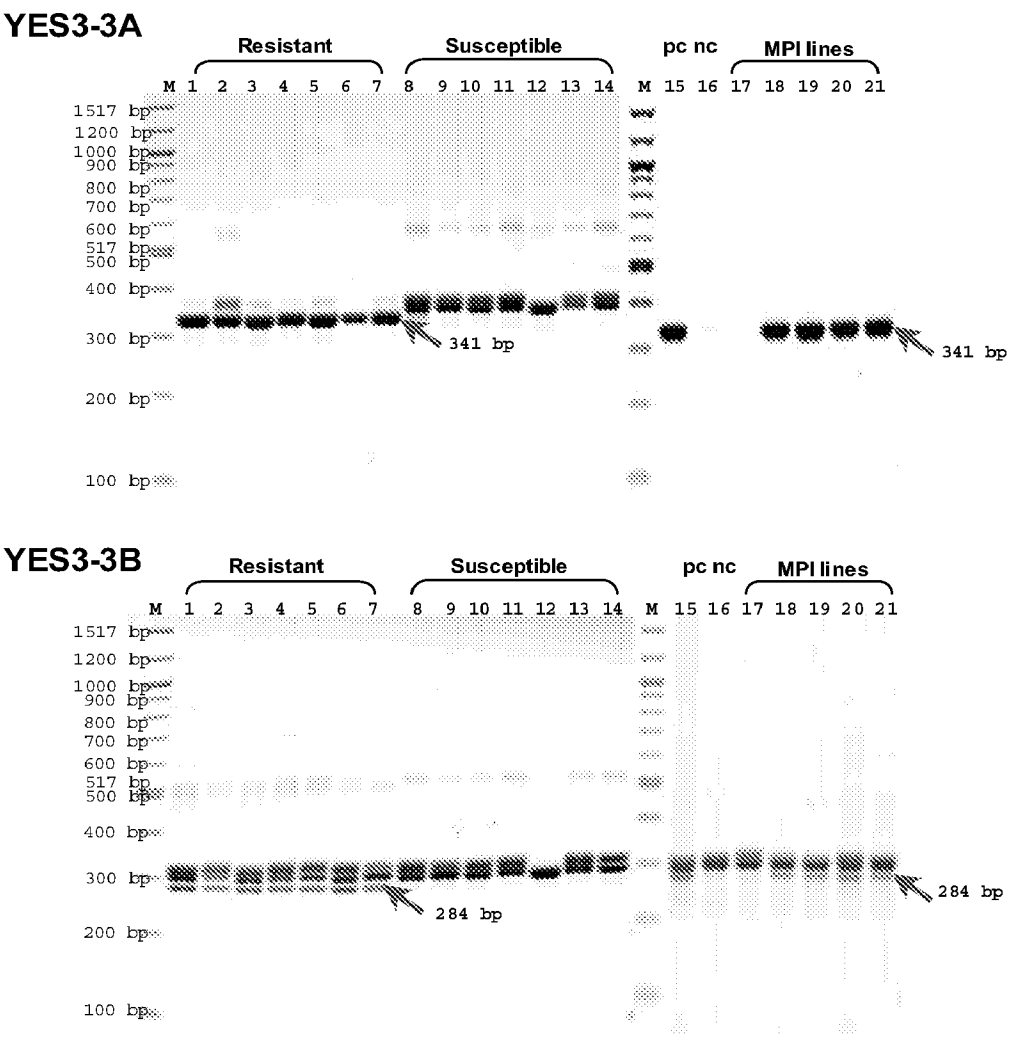


Fig. 1 Developed STS marker analysis in potato varieties and MPI breeding lines. The *arrows* indicate the target fragments for extreme resistance. *Lanes 1* to *7*, extremely resistant cultivars Assia, Barbara, Bettina, Forelle, Jumbo, Maxi and Ute; *lanes 8* to *14*, susceptible cultivars Angela, Bolero, Exempla, Natalie, Panda, Rebecca and Walli; *lanes 15* to *21*, Bettina (*pc* positive control), Ulme (*nc* negative control), MPI 44.1016/10, MPI 50.140/5, MPI 54.4129/288, MPI 55.957/54, MPI 64.956/68, *M*: 100 bp DNA ladder (New England Biolabs, UK)

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Magyar Rozsa, Maxi, Meduza, Nimfy, Oktan, Petra, Pilica, Pirola, Rania, Roeslau, San, Sibu, Solara, Szignal, Tomba, Ute, Wega, and White Lady) and four MPI lines (MPI 50.140/5, MPI 54.4129/288, MPI 55.957/54 and MPI 64.956/68) as extremely resistant to PVY, in alignment with phenotypic data (Table 2). On the other hand, the target fragments of markers YES3-3A and YES3-3B were absent in the cultivars Corine and Sánte, which were reported as extremely resistant potato cultivars by Ross (1986). Both of these two cultivars were identified as the T/β cytoplasm type (Table 2).

Analysis of Plastid-mitochondrial Configurations

Three different plastid-mitochondrial configurations (W/α, W/γ, and T/β) of 188 European potato varieties were identified by ALC_1,3 and ALM_4,5 marker analysis according to Lössl et al. (1999, 2000). Examples of the three different cytoplasm types (W/α, T/β, W/γ) are presented in Fig. 2.

The 38 potato varieties' cultivars were classified as the W/α cytoplasm type, 51 potato varieties were identified as carrying the W/γ cytoplasm type, and 99 potato varieties were found to have the T/β cytoplasm type (Table 2). All 38 extremely resistant potato cultivars presenting STS markers were identified as the W/γ cytoplasm type. All MPI lines were identified with the W plastid type, and only the MPI 44.1016/10 line was found to have the mitochondrial α type, whereas the four extremely resistant MPI lines were determined to carry the mitochondrial γ type.

Comparison of Extreme Resistance to PVY with Mitochondrial Type and Male Sterility

A pollen germination test was performed on 38 field-planted potato cultivars carrying the same W plastid type but different mitochondrial types (α or γ) (Table 2). All 16 potato cultivars carrying the mitochondrial α type (Albatros, Baronesse, Birgit, Bonanza, Calla, Fausta, Marabel, Maxilla, Natalie, Pallina, Panda, Patrona, Ponto, Ramses, Sempra, and Ulme) were identified as having male fertility by observation of pollen germination with a range of different efficiencies due to different genotypes, whereas, only few or hundreds, relative small amounts of pollen grains with wrinkled form and without vitality were obtained in the following 22 potato cultivars with the mitochondrial γ type: Acapella, Amado, Arosa, Assia, Bettina, Forelle, Franzi (1977), Hinga, Jumbo, Klepa, Kuba, Kuras, Logo, Maxi, Meduza, Nimfy, Pasja, Presto, Roberta, Sibu, Solara and Tomba. Among these, 16 potato cultivars were extremely resistant as identified by the Ry_{sto} STS markers YES3-3A and YES3-3B. Figure 3 represents two different pollen types in three potato cultivars with the mitochondrial γ type or α type and a high resistance level to PVY (1): wrinkled pollen grains of the extremely resistant cultivar Jumbo (1*, γ type) and the highly resistant cultivar Logo (1, γ type), and germinated pollen grains of the cultivar Bonaza (1, α type) after incubation for 2 h.

Maternal Pedigree Analysis of Extremely Resistant Cultivars

As extreme resistance to PVY is inherited only by female parents through classical breeding, the maternal pedigree of 33 out of 38 extremely resistant cultivars selected by the Ry_{sto} STS markers YES3-3A and YES3-3B was analyzed in order to trace the source of the resistance gene. Three Hungarian potato cultivars (Magyar Rozsa, Szignal, and White Lady) and two German potato cultivars (Amado and Radia) were excluded from the maternal pedigree analysis because of the deficiency in pedigree information, even though they are known to have the Ry_{sto} gene.

According to the maternal pedigree analysis of extremely resistant cultivars, three groups with different genetic backgrounds could be distinguished depending on the first known crossing parents (Fig. 4). The first group was derived from the crossing of *sto* (ant) with the Erika cultivar. Fifteen potato cultivars belong to this group (Alwara, Arosa, Bettina, Bison, Cordia, Esta, Fanal, Franzi (1977), Heidrun, Jaqueline, Klepa, Meduza, Pirola, Solara and Wega). Two cultivars, Hinga and Nimfy, belong to the second group, which is derived from a cross between stoXIIB and Polonia. The third group has its origin in the crossing parents ((sto hybrid)×Frühmolle×Falke) and Frühmolle, and sixteen potato cultivars belong to this group (Assia, Barbara, Bzura, Forelle, Kuba, Kuras, Jumbo, Maxi, Oktan, Pilica, Petra, Roeslau, San, Sibu, Tomba and Ute). Four marker-selected MPI lines (MPI 50.140/5, MPI 54.4129/288, MPI 55.957/54 and MPI 64.956/68) were ancestors of these extremely resistant potato cultivars as the female parent and Ry_{sto} gene donor in this pedigree.

Discussion

Marker Assisted Selection with an agarose gel system by pyramiding of selectable markers, which are attractive for practical breeding programs, requires less labor and is more feasible and economic than AFLP analysis in polyacrylamide gels. Up to now, more than 25 single dominant genes and 20 R genes have been localized on 12 chromosome maps together with more than 350 markers, which are distributed uniformly and cover approximately 90% of the potato genome (Gebhardt and Valkonen 2001; Barone 2004). Available PCR markers for MAS include: Ry_{adg} (Kasai et al. 2000), Ry_{chc} (Hosaka et al. 2001; Hosaka 2004), *Rx*1 (van der Vossen et al. 2000), *Rx*2 (Bendahmane

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Table 2 Analysis of developed Ry_{sto} STS markers and cytoplasm markers in 188 European potato varieties and MPI lines

Ry_{sto} STS markers present					Ry_{sto} STS markers absent															
W/γ type[a]										W/α type[a]					T/β type[a]							
Cultivar/line	Year	Nation	Response level to PVY[b]	Pollen	Cultivar/line	Year	Nation	Response level to PVY[b]	Pollen	Cultivar/line	Year	Nation	Response level to PVY[b]	Pollen	Cultivar/line	Year	Nation	Response level to PVY[b]	Cultivar/line	Year	Nation	Response level to PVY[b]
Alwara	1985	D	1		Acapella	2000	D	2	S	Albatros	1996	D	1	F	Achat	1976	D	2	Krone	2002	D	2
Amado	2002	D	1	S	Debora	2000	D	4		Artana	1990	D	6		Adora	1988	NL	Medium to high	Lambada	2003	D	1
Arosa	1996	D	1	S	Elkana	1978	NL	High		Artis	1997	D	4		Agria	1985	D	2	Laura	1998	D	1
Assia	1980	D	1*(ER), ER^P	S	Falko	1999	D	5		Astarte	1976	NL	High to very high		Aktivia	2004	D	5	Likaria	1986	D	5
Barbara	1982	D	1*(ER), ER^P		Festien	2000	D	1		Baronesse	1995	D	2	F	Allegra	2003	D	1	Liseta	1988	NL	High
Bettina	1988	D	1*(ER)	S	Fox	1981	D	1		Birgit	1993	D	2	F	Alpha	1925	NL	Medium to high	Lolita	2003	D	2
Bison	1968	D	Very high		Logo	1983	D	1	S	Bonanza	1997	D	1	F	Amigo	1970	D	7	Lubeca	2002	D	1
Bzura	1983	Po	ER^P		Olga	2003	D	1		Calla	1990	D	2	F	Andante	2004	D	4	Marella	2001	D	1
Cordia	1974	D	Very high		Pasja	2000	Po	2	S	Ceza	1986	Po	High		Angela	2000	D	5	Marlen	1999	D	3
Esta	1978	D	1, ER^P		Presto	2001	D	1	S	Colette	1995	D	1		Astoria	1997	D	2	Miriam	1988	D	3
Fanal	1968	D	1, ER^P		Roberta	2002	D	2	S	Combi	1988	D	1		Atica	1971	D	3	Mondial	1987	NL	High
Forelle	1979	D	1*(ER)	S	Solist	1999	D	5		Elfe	2003	D	3		Avano	2005	D	7	Monza	1980	D	1
Franzi	1977	D	1*(ER)	S	Terra	2004	D	1		Fausta	1976	D	3	F	Berber	1983	NL	Medium	Nora	2000	D	1
Heidrun	1978	D	1*(ER), ER^P							Flora	1998	D	2		Bolero	1998	D	1	Pino	1991	D	1
Hinga	1996	Po	ER^P	S						Franca	1987	D	3		Camilla	1998	D	1	Pirol	2000	D	4
Jaqueline	1996	D	1							Franzi	2001	D	2		Candella	2006	D	6	Power	1999	D	3
Jumbo	2002	D	1*(ER)	S						Frühgold	2004	D	2		Carmona	2000	D	2	Priamos	2002	D	6
Kiepa	1997	Po	ER^P	S						Gunda	1999	D	5		Christa	1975	D	6	Producent	1986	D	6
Kuba	1999	Po	1	S						Kartel	1994	NL	Medium		Cicero	1995	NL	High to very high	Quadriga	2005	D	8
Kuras	1996	NL	1	S						Mara	1973	NL	High		Cindy	2002	D	5	Quarta	1979	D	4
Magyar Rozsa	1980	H	Very high, ER^H							Marabel	1993	D	1	F	**Corine**	1976	NL	High	Rebecca	1984	D	2
Maxi	2004	D	1*(ER)	S						Maxilla	1981	D	2	F	Darwina	1981	NL	High	Rheinhort	1959	D	1
Meduza	1997	Po	ER^P	S						Moni	1972	D	2		Desiree	1962	D	2	Rikea	1984	D	2
Nimfy	1998	Po	ER^P	S						Natalie	1995	D	2	F	Ditta	1991	D	2	Rincona	1999	D	2
Oktan	2000	D	1							Palladia	1991	D	3		Draga	1970	NL	Medium to high	Romanze	2004	D	1
Petra	1991	D	1							Pallina	1995	D	4	F	Edelstein	2002	D	1	Salome	2001	D	1
Pilica	1982	Po	Very high, ER^P							Panda	1986	D	3	F	Eigenheimer	1895	NL	Medium to high	Sánte	1983	NL	Very high
Pirola	1976	D	1*(ER), ER^P							Patrona	1994	D	5	F	Exempla	1997	D	3	Satina	1993	D	1
Rania	2001	D	1							Ponto	1984	D	3	F	Exquisa	1992	D	2	Saturna	1970	D	6
Roeslau	1983	D	1*(ER)							Ramses	2004	D	3	F	Fina	1955	D	5	Secura	1985	D	1
San	1980	Po	Very high, ER^P							Renate	1993	D	3		Fontane	1999	NL	Very high	Selma	1972	D	3
Sibu	1993	D	1	S						Rita	1988	D	2		Freya	1998	D	5	Seresta	1994	NL	High
Solara	1989	D	1	S						Ruta	1985	Po	Medium to high		Frieda	1999	D	3	Sieglinde	1953	D	3
Szignal	Uk	H	Very high, ER^H							Scala	1982	D	2		Frisia	1988	NL	Medium	Sira	1986	D	3
Tomba	1995	D	1	S						Sempra	1997	D	2	F	Furore	1930	NL	Medium to high	Sirius	1997	D	5

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Ute	1982	D	1*(ER), ER^P		Sissi	1989	D	1		Gala	2002	D	1		Sirtema	1947	NL	Medium to high
Wega	1978	D	1, ER^P		Tessi	1998	D	2		Gamma	1971	D	Medium to high		Sjamero	1997	D	1
White Lady	Uk	H	Very high, ER^H		Ulme	1991	D	1	F	Gloria	1972	D	6		Sommergold	1987	D	1
										Granola	1975	D	3		Spunta	1968	NL	High
MPI 50.140/5	Uk	D	Very high		MPI 44.1016/10	Uk	D	Uk		Hansa	1956	D	8		Tanja	1967	NL	Medium
MPI 54.4129/288	Uk	D	Very high							Impala	1989	D	4		Tizia	2004	D	1
MPI 55.957/54	Uk	D	Very high							Indira	1981	D	2		Triumpf	2004	D	1
MPI 64.956/68	Uk	D	Very high							Imgard	1960	D	4		Ukama	1977	D	6
										Jaeria	1969	NL	Medium to high		Ulla	1970	D	8
										Jelly	2002	D	2		Valetta	2004	D	7
										Jetta	1967	D	4		Velox	1994	D	4
										Juliane	1999	D	2		Victoria	1997	NL	Medium
										Juwel	2004	D	1		Vienna	2004	D	6
										Kardent	1995	NL	High		Walli	1993	D	3
										Konnoran	2003	D	2					

1 (ER)* indicates registered extremely resistant cultivars by BSA in Germany; the European cultivated potato database (http://www.europotato.org) describes the resistance level to PVY from "low" to "very high", ER^H indicates confirmed Hungarian extremely resistant potato cultivars, ER^P marks Polish extremely resistant potato cultivars (Flis et al. 2005). Boldface indicates extremely resistant potato cultivars identified as carrying the gene Ry_{sto} by Ross (1986).

Uk Unknown, *ER* extreme resistance, *S* sterile, *F* fertile, *D* Germany, *NL* The Netherlands, *H* Hungary, *Po* Poland

[a] Different cytoplasm (plastid-mitochondria) types identified using PCR markers (Lössl et al. 1999, 2000)

[b] Range of resistance level to PVY of German potato cultivars is from "1" to "9" (highly resistant to highly susceptible) according to the Beschreibende Sortenliste Kartoffeln (1979, 1983, 1988, 1993, 1996, 2001, 2003, 2004, 2005, 2006) by Bundessortenamt (BSA), Germany.

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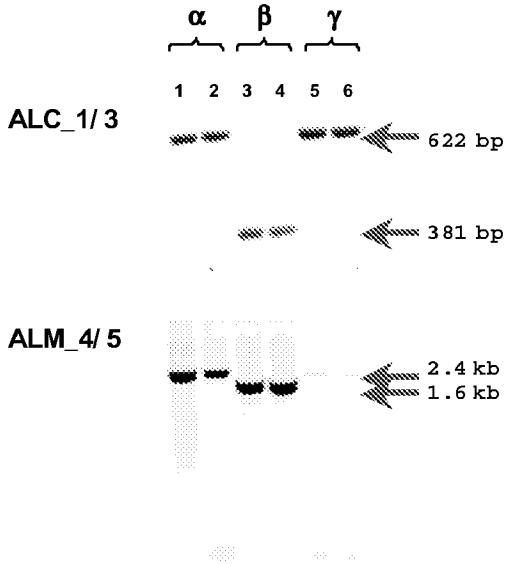


Fig. 2 Determination of three different plastid-mitochondrial configurations in potato cultivars. *Lanes 1* to *6*: Bonanza, Combi, Exquisa, Hansa, Assia, Bettina

et al. 2000), *GroV1* (Jacobs et al. 1996), *Gro1* (Paal et al. 2004), *H1* (Niewöhner et al. 1995), *Gpa2* (van der Vossen et al. 2000), *R1* (Ballvora et al. 2002), *R3a* (Huang et al. 2005) and *Rpi-blb1* (Song et al. 2003). Until now, a precise and feasible PCR marker for the Ry_{sto} gene has not been published. Even considering the precision of the $STM0003_{111}$ marker, a polyacrylamide gel system was required because of the small size of the fragments, and this was also necessary for the AFLP markers. In this study, primers were designed from two different sequence sources that involve whole sequences of one AFLP marker, excluding adapter sequences (24 bp). The right sequence was confirmed by marker analysis of YES3-3A and YES3-3B. Difficulties by the extraction of an AFLP fragment have been already reported by Brugmans et al. (2003), who considered that an extracted AFLP fragment from a polyacrylamide gel contains often multiple fragments, which results from co-isolation of background amplification products. PCR with these primers (3F/3AR (YES3-3A) and 3F/3BR (YES3-3B)) successfully selected extremely resistant potato cultivars out of the tested potato varieties by amplifying different target sizes (341 and 284 bp).

The Ry_{sto} AFLP marker E+ACC/M+CTC-365 is one of 12 AFLP markers linked to the Ry_{sto} gene (Song 2004; Song et al. 2005) together with the SSR marker $STM0003_{111}$, according to Milbourne et al. (1998). However, a BLASTN (nucleotide to nucleotide) analysis of the SOL Genomics Network (http://www.sgn.cornell.edu) did not reveal significant sequence homology of the Ry_{sto} AFLP marker E+ACC/M+CTC-365 or of $STM0003_{111}$ (BV723984) with other members of the database.

Through molecular marker analysis using YES3-3A, YES3-3B, ALC_1,3, and ALM_4,5, all extremely resistant potato cultivars carrying the Ry_{sto} gene were identified as the W/γ cytoplasm type and with male sterility. These results agree with Ross (1986) and Lössl et al. (1999,2000). Ross reported that PVY-immune *S. stoloniferum* back cross hybrids usually function only as females. Lössl et al. (1999, 2000) reported that the mitochondrial γ type is associated with cytoplasmic male sterility. *S. stoloniferum* carrying the Ry_{sto} gene was logically expected to have the mitochondrial γ type due to the pedigree; however, Lössl et al. (1999) clustered the mitochondrial type of *S. stoloniferum* to the α type of the mitochondrial genome, as in our experiments with ten different *S. stoloniferum* lines from the German potato gene bank. The origin of the *Ry* resistance gene in *S. stoloniferum* could be different according to specific accession numbers. Cockerham (1970) has already mentioned a large diversity of *S. stoloniferum* by evaluation of extreme resistance to PVY in the progenies, which were derived from crosses with different *S. stoloniferum* access numbers. He classified the resistance reactions to PVY into the three different phenotypes (Ry_{sto}, Ry_{sto}^{na} and Ry_{sto}^{nl}) among seven different pheno-

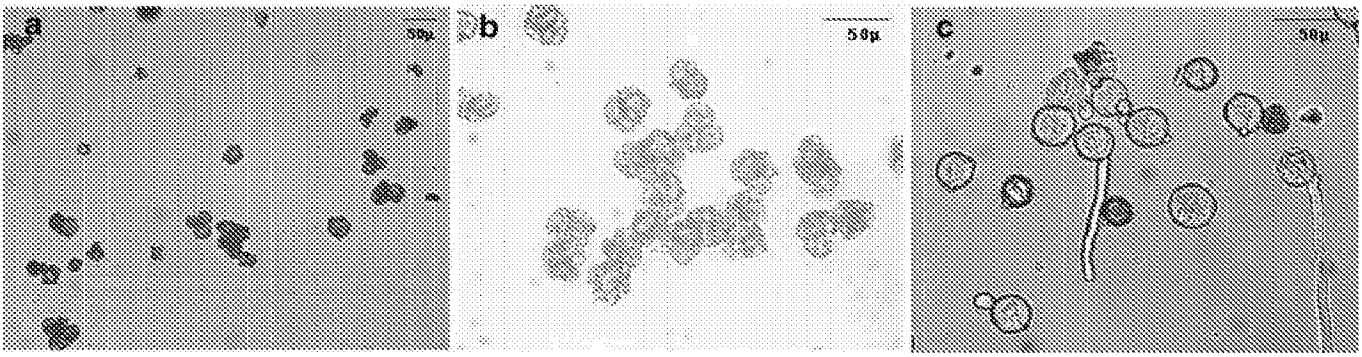


Fig. 3 Pollen grains of potato cultivars with a high resistance level to PVY and different mitochondrial types after 2 h incubation in pollen germination solution. **a** Wrinkled pollen grains of the extremely resistant potato cultivar Jumbo (1*, γ type). **b** Wrinkled pollen grains of the highly resistant cultivar Logo (1, γ type). **c** Elongated pollen grains of the Bonanza cultivar (1, α type)

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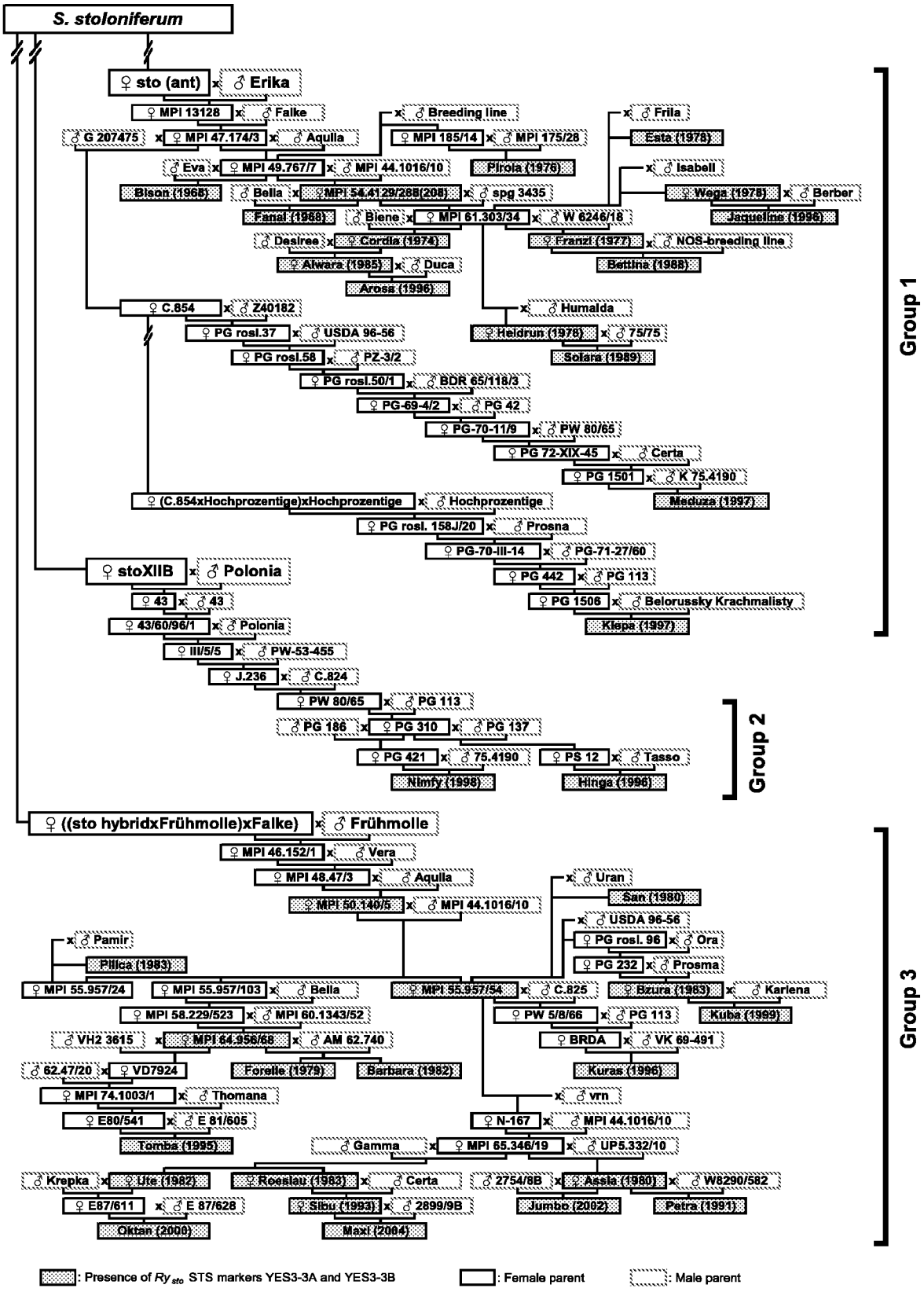


Fig. 4 Maternal pedigree of extremely resistant potato cultivars and breeding lines

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typic responses for the combined reactions of *S. stoloniferum* to virus Y and virus A. The extreme resistance Ry_{sto} represents phenotype 1, and has its known origin in the lines CPC 9, CPC 28.4 and PI 160226. Cockerham's classification also explains the different resistance gene type and origin (Ry_{sto}^{nl}) from the Corine (NL, 1973) and Sánte (NL, 1983) cultivars, which have the mitochondrial β type and absence of the Ry_{sto} STS marker polymorphism. The resistance gene origins of both of these cultivars were derived from the male parents Y 193 and Y 188, descendants of *S. stoloniferum* CPC 2093, which belongs to class 3 of Cockerham (1970). Similarly, two different single dominant *Phytophthora* resistance genes (*Rpi-blb1* (RB), *Rpi-blb2*) from a different accession number of *Solanum bulbocastanum* were reported to be located on chromosome VIII (van der Vossen et al. 2003; Song et al. 2003) and chromosome VI (van der Vossen et al. 2005). It remains unclear whether the γ mitochondria type of the three *S. stoloniferum*-hybrids was introgressed from another parent or if *S. stoloniferum* ancestors had the various mitochondrial genome types (α, β, γ). However, we infer the latter based on our results and references.

In the maternal pedigree analysis, all cultivars and breeding lines identified with the Ry_{sto} markers are extremely resistant to PVY, and are also descendents of MPI breeding lines with an *S. stoloniferum* genetic background, which have been widely spread through Germany and the whole of Europe by classical breeding programs as female parents. All extremely resistant cultivars from Germany belong to Group 1 or Group 3, which are derived from different cross parents. In the Polish cultivars, the extreme resistance to PVY was derived from breeding line MPI 47.174/3, which belongs to Group 1, in the case of Meduza and Klepa, whereas the Nimfy and Hinga cultivars belong to Group 2, a relatively small group with only Polish breeding lines and cultivars. However, there is no information about the Ry_{sto} origin from the ancestors of the three groups in this pedigree analysis. The question of the original source will remain open.

We presume that the five cultivars that have been excluded from pedigree analysis have the same Ry_{sto} gene as the 33 extremely resistant potato cultivars. The extreme resistance of the Rania cultivar is known to be derived from a Polish breeding line. According to the genetic background of the Amado cultivar, its extreme resistance should be derived from the Kuras cultivar as the male parent. No further information exists about the female parent breeding line BH84-212; however, the Kuras cultivar as a male parent is disputable because its male sterility has already confirmed by extensive breeding experiments and its inability to induce pollen germination. It is unclear whether the order of the cross parent was changed, or whether the pedigree is wrong. White Lady is known as one of the extremely resistant potato varieties in Hungary carrying the Ry_{sto} gene, as with Magyar Rozsa, and Szignal. These cultivars confirmed male sterility and extreme resistance, and descended from *S. stoloniferum*, according to J. Taller at the University of Veszprem in Hungary. His group localized the Ry_{sto} gene to the same location, with a 2.1 cM map distance in 120 individuals of the F_1 tetraploid population derived from White Lady by using the marker $STM0003_{111}$ (Taller, J., personal communication).

The comparison of developed STS (Ry_{sto}) marker selection, mitochondrial type analysis, and maternal pedigree confirms that the ancestors of the *S. stoloniferum*-hybrids as resistance gene source (Ry_{sto}) were male sterile. Namely, the extreme resistance gene (Ry_{sto}) has always been accompanied with the mitochondrial γ type of female parent in the classical potato breeding program. Recently, it was possible to break the linkage of extreme resistance to PVY (Ry_{sto}) and cytoplasmic male sterility by protoplast fusion. Fertile male somatic hybrids carrying the Ry_{sto} gene in the nuclei and the mitochondrial α type in cytoplasm could be regenerated in protoplast fusion combinations between a resistant dihaploid genotype to PVY carrying the mitochondrial γ type and a susceptible genotype having the mitochondrial α type (Schwarzfischer, A., unpublished data).

The Ry_{sto} STS markers YES3-3A and YES3-3B were also successfully applied to a MAS program using a 96-deep well DNA preparation for numerous samples in 2006. With these markers, a selective screening method for the most common Ry_{sto} gene in Europe is now feasible.

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Predicted kinase-3a motif of a resistance gene analogue as a unique marker for virus resistance

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Abstract A DNA fragment (ADG2, 310 bp) 77% homologous to the gene *N* (resistance to tobacco mosaic virus in *Nicotiana glutinosa*) and 53% homologous to *RPP5* (resistance to *Peronospora parasitica* in *Arabidopsis thaliana*) was amplified by PCR from the diploid potato genotype 2*x*(v-2)7 that carries the gene Ry_{adg} located on chromosome XI and conferring extreme resistance to potato virus Y(PVY). Sequence comparison revealed that ADG2 spans a region corresponding to the predicted kinase-2 and kinase-3a motifs in *N* and *RPP5*. One of the 12 nucleotide differences detected between ADG2 and a homologous fragment from a PVY-susceptible potato genotype was located within the predicted kinase-3a motif. This single nucleotide substitution of G → C, resulting in an amino-acid substitution Ser → Thr, abolished the *Bbv*I recognition site of ADG2, which was shown to distinguish all tested potato genotypes carrying Ry_{adg} from those lacking this gene, irrespective of the genetic background and ploidy level. This PCR-based resistance marker, developed using a resistance gene analogue as a target, is the first example of a PCR-based marker that is generally applicable for selection of an economically important trait in potato.

Key words Potato virus Y · CAPS · PCR marker · Resistance · Solanum tuberosum

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Introduction

The disease resistance genes isolated from plants contain common features that can be used to group them into different classes (Baker et al. 1997; De Wit 1997; Hammond-Kosack and Jones 1997). Six genes, grouped into two classes, encode predicted cytoplasmic proteins that all contain nucleotide-binding sites (NBS) and leucine-rich repeats, but differ in having either leucine zippers (genes *RPS2*, *RPM1* and *I2*) or Toll and interleukin receptor-like motifs (genes *N*, *L6* and *RPP5*) at their N-terminus.

Conserved motifs of the resistance genes are attractive targets for the design of degenerate primers and the amplification of fragments of unknown resistance genes by the polymerase chain reaction (PCR). Many resistance gene-like fragments (RGL) have been obtained by this technique and mapped to the known positions of resistance genes on chromosomes in important crop species of the families *Solanaceae* (Leister et al. 1996), *Leguminosae* (Kanazin et al. 1996; Yu et al. 1996), *Gramineae* (Leister et al. 1998), *Cichoriaceae* (Shen et al. 1998) as well as in the model species *Arabidopsis thaliana* (family *Brassicaceae*) (Aarts et al. 1998; Speulman et al. 1998), which has provided further evidence that disease resistance genes are clustered on chromosomes.

Leister et al. (1996) have designed primers to two common regions between the gene *N* for resistance to tobacco mosaic tobamovirus derived from *Nicotiana glutinosa* (Whitham et al. 1994) and the gene *RPS2* for resistance to *Pseudomonas syringae* pv *tomato* derived from *A. thaliana* (Bent et al. 1994; Mindrinos et al. 1994) and used them to PCR-amplify RGLs from potato. The 5′-end primer recognizes a predicted kinase-1a (P-loop) motif (GGVGKTT) of a nucleotide-binding site, and the 3′-end primer of a hydrophobic domain (GLPLAL) approximately 160 amino acids downstream, both of which are 100% conserved between the

two genes. The part of gene *N* flanked by these primers contains a kinase-2 motif, 74 amino acids from the P-loop, and a kinase-3a motif, 97 amino acids from the P-loop (Whitham et al. 1994). Three of the several amplified RGLs mapped to the region of chromosome XI (Leister et al. 1996) that is known to contain the gene Ry_{adg} (Hämäläinen et al. 1997, 1998) and another *Ry* gene (Brigneti et al. 1997), both of which control extreme resistance (E) to potato virus Y (PVY) in potato. However, the sequences of these *Ry* genes have not been reported.

The three RGLs mapped to chromosome XI have been re-amplified with new primers specific to each of the three RGLs (Leister 1995) from a diploid potato clone 2*x*(V-2)7 that carries the gene Ry_{adg} for E to PVY. Two of the three RGLs (ADG1 and ADG2) co-segregate with Ry_{adg} in a diploid mapping population produced using 2*x*(V-2)7 as the PVY-resistant parent (Hämäläinen et al. 1998). In the present study, ADG2 was examined for its uniqueness to potato genotypes carrying the gene Ry_{adg}. ADG2 was chosen because it could be PCR-amplified only from potatoes and not from the other solanaceous species tested, in contrast to the other two RGLs mentioned above (our unpublished data). Data showed that ADG2 could be used as an accurate cleaved amplified polymorphic sequence (CAPS) marker (Akopyanz et al. 1992; Konieczny and Ausubel 1993) for Ry_{adg}. To our knowledge this is the first example of a generally applicable PCR-based marker that can be used for the selection of an important trait in potato irrespective of the genetic background and ploidy level.

Materials and methods

Plant material

The cross (77 progeny) made between the diploid ($2n = 2x = 24$) potato breeding line 2*x*(v-2)7 (E to PVY) and the diploid potato clone 84.194.30 (susceptible to PVY), as well as the expression of resistance to PVY in the parental lines and progeny have both been previously described (Valkonen et al. 1994 b; Hämäläinen et al. 1998). E to PVY controlled by *Ry* genes is expressed by the fact that no symptoms are observed and no virus detected by DAS-ELISA following graft-inoculation. Hypersensitive resistance (H) to PVY° controlled by *Ny* genes is expressed by the development of necrotic lesions in top leaves and/or top necrosis in graft-inoculated plants and low but detectable titers of virus in the symptomatic leaves. These definitions follow previous literature (Ross 1986; Jones 1990; Valkonen 1994; Valkonen et al. 1996). Resistance to PVY in the other diploid and tetraploid potato genotypes used in this study has either been reported previously or was tested in this study (see Table 1).

Two or three cuttings of each genotype were side-graft inoculated as previously described with the isolate PVY°-UK that belongs to the ordinary strain group of PVY (Valkonen 1997; Hämäläinen et al. 1998). Experiments were carried out in a growth chamber (Weiss Tekhnik) at 17/19°C (photoperiod 18 h; light intensity 250 $\mu mol\ s^{-1} m^{-2}$, spectrum 380–780 nm). Plants were observed for symptom development and the uppermost fully expanded leaves tested for PVY using DAS-ELISA and polyclonal antibodies to PVY (Boehringer Mannheim, Germany) 26–28 days after graft-inoculation, as previously described (Valkonen 1997).

DNA extraction and cDNA synthesis

Extraction of total DNA was carried out using the method of Fulton et al. (1995). Total RNA was extracted from potato leaves using the RNeasy™ Plant kit (Qiagen Ltd., UK). mRNA was purified from total RNA using the Oligotex™ mRNA kit (Qiagen Ltd.). cDNA was synthesized from 10 µl of mRNA (170–320 ng) using 250 ng of random hexamer (Promega, USA) in a final volume of 12.5 µl. cDNA was denatured at 70°C for 10 min and 4 µl of 5 × first-strand buffer (Promega), 1 µl of 10-mM dNTP and 0.5 µl (20 U) of RNase inhibitor (Boehringer Mannheim) were added. Samples were incubated for 2 min at 37°C, then 2 µl (400 U) of Moloney murine leukemia virus reverse transcriptase (Promega) were added and incubation continued for 1 h at 37°C. The reaction was stopped by placing the tube in a water bath at 70°C for 10 min.

Polymerase chain reaction (PCR)

ADG2 was amplified from the total DNA of the parental potato lines 2*x*(v-2)7 and 84.194.30, total DNA of one extremely resistant and two susceptible progeny lines, and cDNA of one extremely resistant and two susceptible progeny lines using the ADG2-specific primer pair 3.3.3s (5′-ATACACTCATCTAAATTTGATGG-3′) and 3.3.3 as (5′-ACTTAACTGCATCATGTTCAAG-3′) (Leister 1995; Hämäläinen et al. 1998). Amplification was carried out either by a conventional heated-block PCR machine (MJ Research, USA) in thin-walled PCR tubes or by the RapidCycler (Idaho Technologies, USA) in glass capillaries. For amplification using the conventional heated-block PCR machine, the reaction mixture contained 50 µl of 10 mM TrisHCl (pH 8.8), 50 mM KCl, 0.8% Nonidet P40, 4 mM of $MgCl_2$, 0.1 mM of dNTPs, 0.25 µM of primers 3.3.3s and 3.3.3as, 1 U of *Thermus aquaticus* (*Taq*) DNA polymerase (MBI Fermentas) and 50 ng of total genomic DNA or 2 µl of cDNA diluted 1 : 10 with water. Amplification conditions were 2 min at 93°C; 40 cycles with 45 s at 93°C, 45 s at 45°C, 60 s at 72°C; and 5 min at 72°C. For the RapidCycler, the reaction mixture consisted of 15 µl of 10-mM TrisHCl (pH 8.8), 50 mM of KCl, 0.8% Nonidet P40, 4 mM of $MgCl_2$, 500 µg/ml of BSA, 0.2 mM of dNTPs, 0.5 µM of primers 3.3.3s and 3.3.3as, 0.5 U of *Taq* DNA polymerase (MBI Fermentas) and 50 ng of total genomic DNA or 2 µl of cDNA diluted 1 : 10 with water. Amplification conditions were: 30 s at 94°C; 45 cycles with 0 s at 94°C, 0 s at 45°C, and 15 s at 72°C.

For sequencing, the amplified products were purified using a QIAquick PCR Purification Kit (Qiagen Ltd.), cloned in pGEM-T vector (Promega) and sequenced using an automated ALF DNA Sequencer (Pharmacia, Sweden) and a Thermo Sequence Kit (Amersham, UK). Sequence comparisons were carried out using the PCGENE 6.85 software (IntelliGenetics Inc., Switzerland).

Restriction analysis of the PCR products was carried out by precipitating with ethanol (Sambrook et al. 1989) and digesting with selected restriction enzymes in a total volume of 10 µl according to the manufacturer's instructions. The digested PCR products were electrophoretically separated in a 2.0% agarose gel (Eastman Kodak Company, USA) containing 0.1 µg/ml of ethidium bromide in 1 × TBE buffer (Sambrook et al. 1989). Each potato line was tested at least twice.

Results

The PCR product of 0.35 kbp, corresponding to the size of ADG2 (including primer sequences), was amplified

from all the diploid and tetraploid potatoes tested in this study, irrespective of their resistance phenotype, using the primers specific to ADG2. Furthermore, ADG2 was amplified and sequenced from the cDNA of two progeny lines. By contrast, no amplification product was obtained from other solanaceous species, such as eggplant (*Solanum melongena*), tomato (*Lycopersicon esculentum*), tobacco (*Nicotiana tabacum*) and the non-tuber-bearing potato species *Solanum brevidens* (data not shown).

Sequence comparison between the previously characterized ADG2 fragment amplified from the PVY-resistant parental line 2*x*(V-2)7 (EMBL Nucleotide Sequence Database Accession No. Y15297) and the ADG2 fragment of the PVY-susceptible parental line 84.194.30 characterized in this study (EMBL Nucleotide Sequence Database Accession No. Y17789) showed that both ADG2 fragments were of the same size (310 bp, excluding primer sequences) and contained 12 nucleotide differences. Sequencing of the amplified products from five progeny lines (including the cDNA of two of them) showed that they contained either both sequences (heterozygous progeny expressing E to PVY) or the ADG2 of the susceptible parent (PVY-susceptible progeny), as expected (data not shown). The fact that ADG2 was amplified from purified mRNA pools and contained no translation stop codon indicated that it is part of a transcribed gene.

In order to develop a CAPS marker, the PCR products obtained using the RapidCycler and glass capillaries under the optimized conditions were digested with selected restriction enzymes. This analysis was first carried out on the 77 progeny of the diploid mapping population. Two restriction enzymes, *Msp*I (MBI Fermentas, Lithuania) and *Bbv*I (New England Biolabs, USA), provided useful CAPS markers. *Msp*I had a single, unique restriction site in the ADG2 fragment of the PVY-resistant parental line, whereas *Bbv*I had a single, unique restriction site in the ADG2 fragment of the susceptible parental line. The recognition site of *Bbv*I was located within the predicted kinase-3a motif (Fig. 1). In the ADG2 of 2*x*(v-2)7, a single nucleotide substitution G → C that abolishes the *Bbv*I recognition site results in an amino-acid substitution, Ser → Thr, in the variable part of the kinase-3a consensus motif [(E, A, G, P, F)(T, S, G, F)XXX(Y, R) where X is a variant amino-acid residue (Traut 1994)] (Fig. 1).

*Msp*I (1 U) specifically cut the PCR product of 2*x*(v-2)7 and of progeny lines carrying Ry_{adg}, resulting in one visible band (317 bp, cut) in 2*x*(v-2)7, one visible band (355 bp, uncut; includes the primers at both termini) in 84.194.30 and PVY-susceptible progeny, and two visible bands (317 bp and 355 bp; cut and uncut, respectively) in the heterozygous PVY-resistant progeny. The smaller cleavage product of 38 bp was not visible in the gel. The CAPS marker band (317 bp) was observed in all progeny expressing E to PVY but was not observed in any other progeny line.

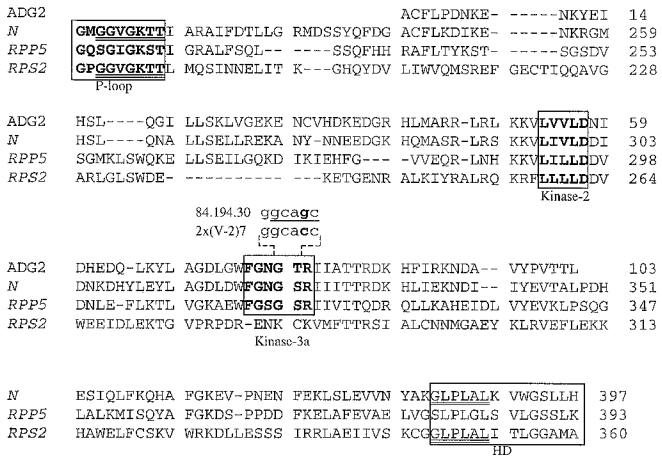


Fig. 1 Comparison of predicted amino-acid sequences of the resistance gene-like fragment ADG2 [PVY-resistant parental line 2*x*(v-2)7] and the genes *N*, *RPP5* and *RPS2*. Amino acids of *N* (Whitham et al. 1994), *RPP5* (Parker et al. 1997) and *RPS2* (Bent et al. 1994; Mindrinos et al. 1994) are *numbered* according to the original reports. The consensus sequences between *N* and *RPS2* used as primer target sites by Leister et al. (1996) are *double underlined*. The predicted P-loop, kinase-2 and kinase-3a motifs are *bold and boxed*, and a predicted hydrophobic domain (HD) is *boxed*. The single nucleotide difference between the ADG2 sequence of 2*x*(v-2)7 (EMBL Nucleotide Sequence Databank Accession No. Y15297) and the PVY-susceptible parental line 84.194.30 (EMBL Nucleotide Sequence Database accession No. Y17789) within the predicted kinase-3a motif is indicated and the *Bbv*I recognition site (gcagc) is *underlined*

With *Bbv*I (0.6 U), the ADG2 of the resistant parent 2*x*(v-2)7 remained uncut (355 bp), whereas the ADG2 of the susceptible parent was cut resulting in a smaller visible band (approximately 270 bp) (Fig. 2). The CAPS marker band (355 bp) was observed in all progeny expressing E to PVY but was not present in any other progeny line. A faint, uncut band could be observed in some progeny that did not carry Ry_{adg}. Digestion with *Bbv*I worked most consistently after precipitation and re-suspension of the PCR product.

Subsequently, the CAPS markers created with *Msp*I and *Bbv*I were tested on 50 selected potato cultivars and breeding lines with different ploidy levels and diverse genetic backgrounds (Table 1). *Msp*I digestion could not distinguish between genotypes carrying and lacking Ry_{adg} (data not shown). In contrast, digestion with *Bbv*I produced the CAPS marker (uncut ADG2 fragment) only in those genotypes (irrespective of their ploidy and pedigree) that express E to PVY and carry the gene Ry_{adg} derived from *Solanum tuberosum* subsp. *andigena* (Fig. 2, Table 1). For example, the potato lines 2*x*(v-3)30, 86.61.26, F1.1, IvP35 and cv Ute, which express E to PVY but in which resistance is not derived from *S.t. andigena* (Valkonen et al. 1994, 1995; Valkonen and Palohuhta 1996), were not recognized with the CAPS marker (Table 1).

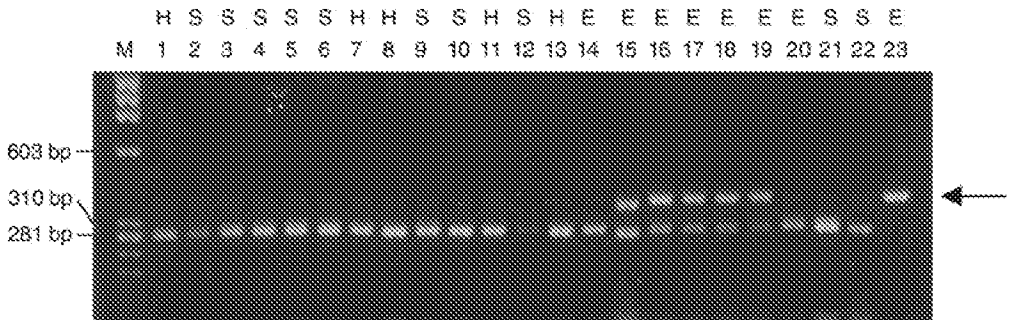


Fig. 2 ADG2 homologues amplified by PCR from independent potato cultivars and breeding lines and subsequently digested with *Bbv*I. Resistance phenotypes are indicated above the lanes: *E, H and S* indicate extreme resistance, hypersensitive resistance and susceptibility to PVY, respectively. The CAPS marker band (non-digestible with *Bbv*I) is indicated by an *arrowhead.* For the pedigree of genotypes analyzed in *lanes 1–21*, see Table 1. Note that the potato lines Ute and IvP35 express extreme resistance to PVY but they do not contain the gene Ry_{adg} according to the pedigree and RFLP marker data. — *Bsu*RI digested ϕX174 DNA; *1* Allegany; *2* Amazone; *3* Andover; *4* Atlantic; *5* Belrus; *6* Bintje; *7* Desirée; *8* Jemseg; *9* King Edward; *10* Maris Piper; *11* Fambo; *12* NY99; *13* TA1.22.5.1; *14* Ute; *15* N140-201; *16* TA3.5.3.6; *17* TA3.5.3.7; *18* TA3.8.3.3; *19* TA3.8.3.4; *20* IvP35; *21* J-40. *Lanes 22 and 23* contain the PVY-susceptible parental line 84.194.30 and PVY-resistant parental line 2*x*(v-2)7, respectively, of the diploid mapping population

In potato line 7XY.1, the CAPS marker was observed in some but not all repeated tests, whereas in those TA clones that contain Ry_{adg} derived from 7XY.1 (Table 1) the CAPS marker was consistently observed. Sequencing of the regions flanking the ADG2 sequence (data not shown), including the primer target sites, showed that the ADG2 sequence of 2*x*(v-2)7 contains one and two nucleotide differences in the target sites of the 5′ and 3′ primers, respectively, as compared to the primer sequences of Leister (1995) used in this study. It is therefore hypothesized that a *Bbv*I-digestible ADG2 homologue more similar to the original ADG2 homologue reported by Leister (1995), and thus preferentially amplified, may exist in 7XY.1 (to be verified in a future study). We recommend that the CAPS marker described in this study should be produced by amplification with primers 5′-ATACTCTCATCTAAATTTGATGG-3′ and 5′-ACTGAACAGCATCATGTTCAAG-3′ that match precisely with our ADG2 sequence. Our recent data (H. Cabrera, S. Vidal and J.P.T Valkonen, unpublished) show that amplification of the genomic clone of the putative gene of which ADG2 (access. no. Y15297) is a part, using primers specific to the 5′ and 3′ termini of the corresponding cDNA clone, results in amplification of a product (approximately 7 kb) only from those potato lines that contain the gene Ry_{adg}.

Discussion

The CAPS marker reported in this study is to our knowledge the first example of a PCR-based marker that is generally applicable for the selection of an important trait in potato at different ploidy levels and in diverse genetic backgrounds. Extreme resistance to PVY conferred by the *Ry* genes provides the most effective means to protect potato crops against the significant yield losses that PVY causes in all potato production areas (Hooker 1981; Ross 1986). Therefore, the CAPS marker developed in this study should represent one of the most valuable breeding markers for use with potato. PCR markers have been developed for some other resistance genes in potato: to *R1* that confers resistance to *Phytophora infestans* (Meksem et al. 1995), *Gro*I and *H1* that confer resistance to the root cyst nematode (Ballvora et al. 1995; Niewöhner et al. 1995), and *Nb* that confers hypersensitive resistance to potato X potexvirus (De Jong et al. 1997). However, the markers for *Gro1* and *H1* seem to precisely detect resistant genotypes only in the mapping population in which they have been developed and, thus, are similar to the CAPS marker created with *Msp*I in this study. The applicability of the markers for *R1* and *Nb* in potato material other than the original mapping population is not clearly demonstrated.

Use of a CAPS marker is technically easier, and the amount of DNA needed is much smaller, than an RFLP analysis of PVY resistance (Hämäläinen et al. 1998). Even with an expensive restriction enzyme such as *Bbv*I the cost of a CAPS analysis is only about a half the cost of an RFLP analysis. In CAPS analysis, a PCR product is always obtained and its quantity can be assessed, which increases reliability. On the other hand, the use of primers that recognize a sequence or a single nucleotide difference (Sawa et al. 1997) specific to the resistant genotype, and result in an amplification product only from resistant genotypes, avoids the restriction-analysis phase and makes the test simpler. Development of the latter type of marker based on the ADG2 sequence is currently in progress.

This study has shown that the resistance gene-like fragments obtained by PCR on the basis of common sequence motifs of known resistance genes can be useful starting points for the development of accurate PCR-based markers for uncharacterized resistance genes. It is intriguing why the restriction analysis of ADG2 using *Bbv*I was so precise in detecting genotypes that carry the gene Ry_{adg}, taking into consideration that a product corresponding to the size of ADG2 was

Table 1 Potato cultivars and breeding lines tested with the CAPS marker created by PCR-amplification and subsequent *Bbv*I digestion of ADG2

Potato clone	Ploidy	Background[a]	Resistance expression[b]	CAPS marker	References to pedigree and PVY resistance
Allegany	4*x*	*tbr*, *adg*	H	–	R.L. Plaisted (pers com); Valkonen et al. 1994 b
Amazone	4*x*	*adg*	S	–	Stegemann and Schnick 1985; Valkonen and Palohuhta 1996
Andover	4*x*	*adg*, *tbr*, *chc*	S	–	Plaisted et al. 1998; Anon. 1994
Atlantic	4*x*	*adg*, *tbr*, *chc*	S	–	R.L. Plaisted (pers com); Valkonen et al. 1994 a
Belrus	4*x*	*tbr*	S	–	Hämäläinen et al. 1997
Bintje	4*x*	*tbr*	S	–	Stegemann and Schnick 1982; Valkonen and Palohuhta 1996
Desirée	4*x*	*tbr*	H	–	Stegemann and Schnick 1982; Jones 1990
E74-7	4*x*	*adg*	E	+	R.L. Plaisted (pers com); Hämaläinen et al. 1997
Fambo	4*x*	*tbr*, *adg*, *dms*	H	–	Stegemann and Schnick 1982; Valkonen and Palohuhta 1996
Jemseg	4*x*	*tbr*	H	–	Valkonen et al. 1994 a
Katahdin	4*x*	*tbr*	H	–	Stegemann and Schnick 1982; Jones 1990
King Edward	4*x*	*tbr*	S	–	Stegemann and Schnick 1982; Jones 1990
Maris Piper	4*x*	*adg*, *dms*	S	–	Stegemann and Schnick 1982; Jones 1990
Matilda	4*x*	*tbr*, *dms*	H	–	Valkonen and Palohuhta 1996
Monona	4*x*	*tbr*	S	–	Anon. 1994
N140-201	4*x*	*tbr*, *ber*, *adg*	E	+	R.L. Plaisted (pers com); Hämäläinen et al. 1997
Nicola	4*x*	*adg*, *dms*	H	–	Stegemann and Schnick 1982; Valkonen and Palohuhta 1996
Norchip	4*x*	*tbr*	S	–	R.L. Plaisted (pers com); Anon. 1994
NY99	4*x*	*adg*, *tbr*	S	–	R.L. Plaisted (pers com); Hämäläinen et al. 1997
Pentland Dell	4*x*	*tbr*, *dms*, *phu*	H	–	Stegemann and Schnick 1982; Valkonen et al. 1994 a
Pentland Ivory	4*x*	*tbr*, *chi*, *dms*, *phu*	H	–	Stegemann and Schnick 1982; Jones 1990
Pito	4*x*	*tbr*	H	–	Stegemann and Schnick 1982; Valkonen 1997
Posmo	4*x*	*tbr*	H	–	Stegemann and Schnick 1982; Valkonen and Palohuhta 1996
Q237-8	4*x*	*tbr*, *adg*, *ver*	E	+	R.L. Plaisted (pers com); Hämäläinen et al. 1997
Record	4*x*	*tbr*	H	–	Stegemann and Schnick 1982; Valkonen and Palohuhta 1996
Rosamunda	4*x*	*adg*, *dms*, *phu*	H	–	Stegemann and Schnick 1982; Valkonen and Palohuhta 1996
S48-6	4*x*	*adg*, *tbr*	H	–	R.L. Plaisted (pers com); this study
Sebago	4*x*	*tbr*	H	–	Stegemann and Schnick 1982; Hämäläinen et al. 1997
Semena	4*x*	*adg*	H	–	Stegemann and Schnick 1985; this study
Superior	4*x*	*tbr*	S	–	Anon. 1994
7XY.1	4*x*	*adg*	E[c]	+/–	Watanabe et al. 1992
TA1.27.1-1	4*x*	*tbr*, *adg*, *acl*, *chc*, *yun*, *phu*	S[c]	–	K.N. Watanabe (unpublished); Hämäläinen et al. 1997
TA3.2.2	4*x*	*tbr*, *adg*, *acl*, *chc*, *yun*, *phu*	E[c]	+	K.N. Watanabe (unpublished)
TA3.5.3.2	4*x*	*tbr*, *adg*, *acl*, *chc*, *yun*, *phu*	E[c]	+	K.N. Watanabe (unpublished)
TA3.5.3.6	4*x*	*tbr*, *adg*, *acl*, *chc*, *yun*, *phu*	E[c]	+	K.N. Watanabe (unpublished); this study
TA3.5.3.7	4*x*	*tbr*, *adg*, *acl*, *chc*, *yun*, *phu*	E[c]	+	K.N. Watanabe (unpublished); this study
TA3.8.3.2	4*x*	*tbr*, *adg*, *acl*, *chc*, *yun*, *phu*	E[c]	+	K.N. Watanabe (unpublished)
TA3.8.3.3	4*x*	*tbr*, *adg*, *acl*, *chc*, *yun*, *phu*	E[c]	+	K.N. Watanabe (unpublished); this study
TA3.8.3.4	4*x*	*tbr*, *adg*, *acl*, *chc*, *yun*, *phu*	E[c]	+	K.N. Watanabe (unpublished); this study
Torva	4*x*		H	–	K.N. Watanabe (unpublished); this study
Ute	4*x*	*acl*, *sto*, *vrn*	E[d]	–	Stegemann and Schnick 1985; Valkonen and Palohuhta 1996
Valtti	4*x*	*tbr*, *dms*	H	–	Stegemann and Schnick 1982; Valkonen 1997
Van Gogh	4*x*		S	–	Valkonen and Palohuhta 1996
Yukon Gold	4*x*	*tbr*, *phu*	H	–	Johnston and Rowberry 1981; Valkonen 1997
TA1.22.5.1	2*x* + 1[e]	*tbr*, *adg*, *acl*, *chc*, *yun*, *phu*	H	–	K.N. Watanabe (unpublished); this study
2*x*(V-3)30	2*x*	*adg*, *sto*, *tbr*, *phu*	E[d]	–	Watanabe et al. 1994
86.61.26	2*x*	*phu*, *spl*, *stn*, *sto*, *tbr*	E[d]	–	Watanabe et al. 1994; Valkonen et al. 1994 b
F1.1	2*x*	*chc*, *phu*	E[d]	–	Hosaka and Hanneman 1994; this study
IvP 35	2*x*	*phu*	E[d]	–	Hermsen and Verdenius 1973; Valkonen et al. 1995
J-40	2*x*	*phu*, *spl*, *stn*	S	–	Watanabe et al. 1994

[a] *S. t. andigena* (*adg*), *S. t. tuberosum* (*tbr*), *S. phureja* (*phu*), *S. stoloniferum* (*sto*), *S. chacoense* (*chc*), *S. sparsipilum* (*spl*), *S. stenotomum* (*stn*), *S. demissum* (*dms*), *S. chiloense* (*chi*), *S. acaule* (*acl*), *S. vernei* (*vrn*), *S. yungasense* (*yun*), *S. berthaultii* (*ber*), *S. verrucosum* (*ver*). More detailed information about the pedigrees can be obtained from authors

[b] E, extremely resistant; H, hypersensitive; S, susceptible

[c] TA (*tuberosum-acaule*) clones were produced using the potato line AA-3 (7XY.1 × *S. acaule* 954.3CA) as the female parent (TA3.2.2 and TA3.5.3.2) or male parent (other TA clones) in crosses with an interspecific hybrid DG81.68. E to PVY in the TA clones is conferred by Ry_{adg} derived from 7XY.1 (except TA1.27.1-1 that is susceptible to PVY)

[d] Extreme resistance to PVY not derived from *Solanum tuberosum* subsp. *andigena*

[e] Trisomic

Aardevo Exhibit 1010
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amplified from all tested potato genotypes. ADG2 of 2x(v-2)7 shows 77% nucleotide-sequence homology with the corresponding region of the gene *N* (Whitham et al. 1994) and 53% homology with *RPP5*, a gene for resistance to *Peronospora parasitica* in *A. thaliana* (Parker et al. 1997). *N* and *RPP5* belong to the same class of resistance genes (Baker et al. 1997; De Wit 1997; Hammond-Kosack and Jones 1997) and are the only reported resistance genes with which ADG2 showed >50% sequence homology. Amino-acid sequence comparisons showed that a hypothesized transcribed gene, of which ADG2 is a part of, contains the predicted kinase-2 and kinase-3a motifs (Traut 1994) found in the corresponding region of *N* and *RPP5* (Fig. 1). The *Bbv*I recognition site is located within the predicted kinase-3a motif. The experimental evidence of this study indicates that the nucleotide substitution G → C that abolishes the unique *Bbv*I recognition site and results in the amino-acid substitution (Ser → Thr) at this position is unique to those potato genotypes that carry Ry_{adg}. Because a kinase motif can be the specificity determinant of a resistance gene (Frederick et al. 1998), the interesting possibility remains that the ADG2 fragment used as a marker in this study may be part of Ry_{adg}. However, it may also be part of another closely linked gene located in the same resistance gene cluster on chromosome XI (Leister et al. 1996; Hämäläinen et al. 1998), and these two alternatives need to be examined in a future study.

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The $R_{Pi\text{-}mcd1}$ Locus from *Solanum microdontum* Involved in Resistance to *Phytophthora infestans*, Causing a Delay in Infection, Maps on Potato Chromosome *4* in a Cluster of NBS-LRR Genes

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The distinction between field resistance and resistance based on resistance (*R*) genes has been proven valid for many plant–pathogen interactions. This distinction does not seem to be valid for the interaction between potato and late blight. In this study, a locus involved in late blight resistance, derived from *Solanum microdontum*, provides additional evidence for this lack of distinction. The resistance is associated with a hypersensitive response and results in a delay of infection of approximately 1 to 2 weeks. Both a quantitative as well as a qualitative genetic approach were used, based on data from a field assay. Quantitative trait locus (QTL) analysis identified a QTL on chromosome *4* after correction of the resistance data for plant maturity. A qualitative genetic analysis resulted in the positioning of this locus on the short arm of chromosome *4* in between amplified fragment length polymorphism marker pCTmACG_310 and cleaved amplified polymorphic sequence markers TG339 and T0703. This position coincides with a conserved *Phytophthora R* gene cluster which includes $R2$, $R_{2\text{-}like}$, $R_{Pi\text{-}blb3}$, and $R_{Pi\text{-}abpt}$. This implies that $R_{Pi\text{-}mcd1}$ is the fifth *R* gene of this nucleotide-binding site leucine-rich repeat cluster. The implications of our results on *R*-gene-based and field resistance are discussed.

The disease late blight, caused by the oomycete pathogen *Phytophthora infestans*, is a serious worldwide threat to potato cropping. In order to reduce the damaging effects from *Phytophthora* infection, the use of genetically resistant plants is a potentially viable approach for disease management (Strange and Scott 2005). Eleven resistance (*R*) genes originating from the wild species *Solanum demissum* have been discovered in the last century: *R1, R2, R3*, and *R4* by Black and associates (1953); *R5* and *R6* by Eide and associates (1959); *R7, R8*, and *R9* by Malcolmson and Black (1966); and *R10* and *R11* by Malcolmson (1969). All these resistance genes offer race-specific hypersensitive resistance but have been proven not to be durable (Malcolmson and Black 1966).

To look for new sources of resistance that potentially could be more durable, researchers have turned to other wild potato species besides *S. demissum*. The resistance gene $R_{Pi\text{-}ber}$ from *S. berthaultii* mapped on chromosome *10* (Ewing et al. 2000; Rauscher et al. 2006). $R_{Pi\text{-}ber}$ proved to be a new *R* gene after testing with a set of *Phytophthora* strains capable to identify each of the *R* genes *R1* to *R11*. Four *R* genes have been identified in the wild potato species *S. bulbocastanum*. The *RB* locus has been mapped on chromosome *8* by Naess and associates (2000). One allele of this locus has been cloned by Song and associates (2003) and another allele, $R_{Pi\text{-}blb1}$, by van der Vossen and associates (2003). Another gene that was cloned from *S. bulbocastanum* was $R_{Pi\text{-}blb2}$, located on chromosome *6* (van der Vossen et al. 2005). These two genes, $RB/R_{Pi\text{-}blb1}$ and $R_{Pi\text{-}blb2}$, have tentatively been described as "broad-spectrum resistance genes." This new terminology may imply that a larger number of elicitors is being recognized, or may just indicate the current absence of a single compatible race. Finally, two race-specific resistance genes, $R_{Pi\text{-}blb3}$ and $R_{Pi\text{-}abpt}$, have been mapped to chromosome *4* (Park et al. 2005a and b).

In addition to the dominant *R*-gene-based resistance conferring the hypersensitive response (HR), the so-called field resistance also is known. This type of resistance is race nonspecific, quantitative, and considered to be polygenically inherited. It has been assumed that this type of resistance is more durable than *R*-gene-based resistance. Race-nonspecific foliage resistance is currently differentiated in either maturity-corrected resistance (Bormann et al. 2004), or maturity-associated resistance, as was shown by Toxopeus (1958), Van Eck and Jacobsen (1996), Collins et al. (1999), Oberhagemann et al. (1999), Costanzo et al. (2005), and Visker et al. (2005). The general importance of a locus near marker GP21 on chromosome *5* involved in both maturity and maturity-related resistance was reviewed by Simko (2002).

In addition to *S. bulbocastanum* and *S. berthaultii*, the late blight resistance offered by *S. microdontum* has been used in commercial potato breeding during the last decade, although little is known about its inheritance. Resistance derived from *S. microdontum* genotype MCD167 (accession BGRC 24981/CGN20597) has initially been studied using area under the disease progress curve (AUDPC) data from a field trial (Sandbrink et al. 2000). Their analysis of the quantitative inheritance of this resistance launched two assumptions, that the *S. microdontum* resistance i) was polygenic and ii) conferred race-nonspecific resistance. Sandbrink and associates (2000) identified a major quantitative trait locus (QTL) on chromosome *4* explaining 40% of the phenotypic variation,

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and two QTL on chromosome *5*. An additional major QTL was identified in the same study in genotype MCD 178 (accession BGRC 24981/CGN20597). Bisognin and associates (2005) have also described a field resistance derived from *S. microdontum* (BGRC 27353/CGN20640); however, the linkage group containing the only QTL could not be assigned to a specific potato chromosome. Last, it is known that the foliage resistance observed in *S. microdontum* BGRC 18302 (CGN21342) is also effective in the tuber (Park et al. 2005d)

In this article, we describe the genetic and phenotypic analysis of late blight resistance derived from *S. microdontum*. Our plant material represents a different accession than the accessions described by Sandbrink and Bisognin. The accession used in this study is also used in commercial breeding programs in the Netherlands and consistently displays a 1- to 2-week delay in infection in the field (*unpublished data*). Furthermore, we have mapped our locus on chromosome *4* and narrowed down its position to a conserved *Phytophthora R* gene cluster which includes *R2*, $R_{2\text{-}like}$, $R_{Pi\text{-}blb3}$, and $R_{Pi\text{-}abpt}$.

RESULTS

In vitro and detached-leaf assay.

Because of its efficiency and previous successes, the in vitro late blight resistance assay (Huang et al. 2005) was used to classify 371 seedlings of mapping population RH94-076. The susceptible control Bintje sporulated after 3 days. The resistant control 707TG11-1 showed an HR and no sporulation. Most of the genotypes of RH94-076 were sporulating within 7 days. In all, 128 genotypes sporulated without an HR and 186 genotypes showed a weak, incomplete HR before sporulation. At the end of the observation period, 12 genotypes showed no symptoms and 45 genotypes gave an HR and no sporulation; therefore, those 57 could be considered as resistant. These results indicated that some form of resistance is present. Based on these observations, unambiguous classification of seedlings as resistant or susceptible was impossible. The most important problem was that no apparent Mendelian segregation ratio could be identified, preventing a BSA approach. For these reasons, we proceeded with a detached-leaf assay.

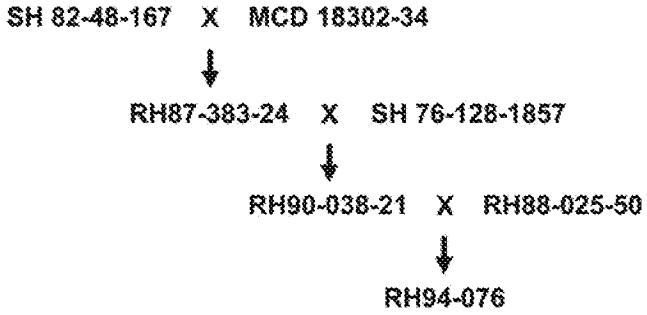


Fig. 1. Pedigree of the *Solanum microdontum*-derived mapping population RH94-076, derived from accession BGRC 18302, backcrossed with susceptible diploid *S. tuberosum* clones.

In the detached-leaf assay, the *S. microdontum*-derived BC1 parent RH90-038-21 was resistant, showing HR, and the *S. tuberosum* parent RH88-025-50 was susceptible. However, clones of the mapping population showed inconsistent phenotypes, where sometimes only one or two leaves of the four leaves per genotype were infected and showed sporulation, whereas the others were without symptoms. When the entire detached-leaf assay was repeated, again with four leaves per genotype, the newly obtained results poorly correlated with the first assay. The phenotypic differences observed among the progeny displayed a continuous distribution and could not be differentiated in a resistant or susceptible class. The resistance derived from *S. microdontum* appeared to be quantitative in nature, which could be much better evaluated using a field experiment. In summary, the value of the in vitro assay and the detached-leaf assay is limited, and only allows us to conclude that these tests require plant material with strong effect *R* genes, as shown before (Huang et al. 2005; Vleeshouwers et al. 1999).

Quantitative genetic analysis of disease resistance and plant maturity.

The mapping population (Fig. 1), consisting of 224 clones, was evaluated for the level of resistance in a field test in 2001. Observations ranged from 7 until 29 days postinfection (dpi). When rAUDPC (relative area under disease progress curve) was used as trait value in Kruskal-Wallis-based QTL detection, three QTL peaks were identified near markers eATGmCAG_235 (at

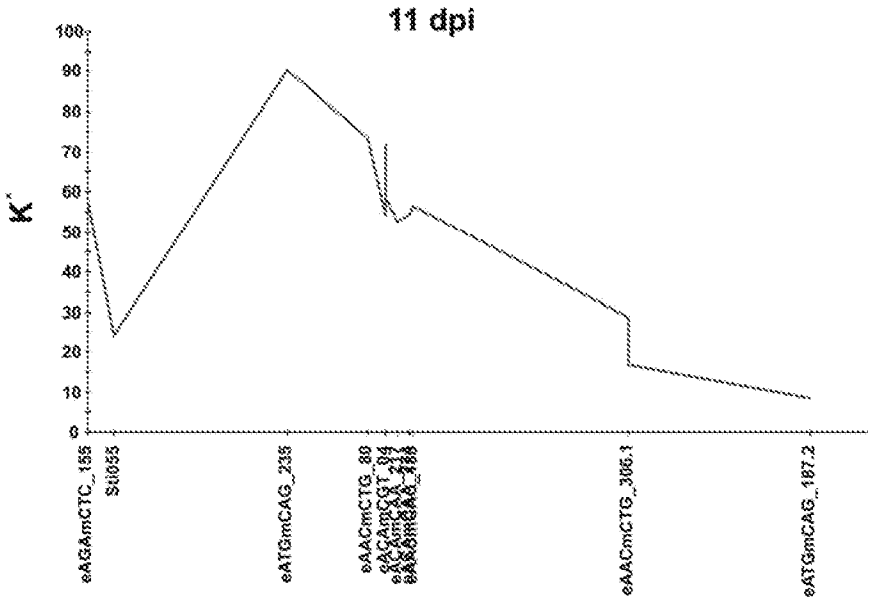


Fig. 2. Kruskal-Wallis curve showing the quantitative trait loci peak at the position of marker eATGmCAG_235 on chromosome *4* of *Solanum microdontum* parent (BC1) RH90-038-21, marking a locus with a major effect on percentage of leaf area infected by *Phytophthora infestans*.

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chromosome *4*), eACAmCAC_19, and Sti032 (both at chromosome *5*) with K* values of 78, 28, and 24, respectively. When percentage of infected foliage at a given day was used as trait value, higher K* values were obtained, with QTL peaks at the same three markers.

The most prominent QTL was identified by any of the markers along maternal chromosome *4*. Amplified fragment length polymorphism (AFLP) marker eATCmCAG_235 identified this QTL regardless of observation time during the whole time range of the experiment. The percentage of infected foliage at 9 to 11 dpi was the most informative trait value, showing the highest K* values (K* = 90 to 91) at AFLP marker eATGmCAG_235. At these days (9 to 11 dpi), all associations between the trait value and the markers of chromosome *4* reached significance levels of $P < 0.0001$, except for the most distal marker eATGmCAG_187 ($P < 0.005$). The position of the Kruskal-Wallis-identified QTL peak at 11 dpi is illustrated in Figure 2. With interval mapping using the angular transformed data, the explained variance was 50.3% at a log of the likelihood ratio (LOD) of 34, indicating a QTL with a large effect, located on chromosome *4*. From 13 dpi, the test statistic lowered gradually from K* = 86 at 13 dpi to K* = 31 at 29 dpi (end of observation). At 29 dpi, the QTL effect resulted in an explained variance of 5.5% at a LOD of 2.88. Observations later than 11 dpi did not offer the same power to detect this QTL because, at later observations, an increasingly larger part of the population was completely infected.

Two other QTL involved in percent foliage infection were found, namely on chromosome *5* of the maternal and the paternal map. The QTL on maternal chromosome *5* of RH90-038-21 had significant ($p < 0.0001$) association with two AFLP markers and Sti032 marker with a K* value of 17 increasing gradually to the highest value, K* = 32, at 25 dpi. With interval mapping, the QTL increased gradually to the highest value of LOD 8.85 with an explained variance of 16.2% at 25 dpi. The QTL on paternal chromosome *5* of RH88-025-50 had significant ($P < 0.0001$) association with three AFLP markers and SSR markers STM5148 and Sti032. From 7 dpi, the K* value of 19 increased gradually to the highest K* = 39 value at the final observation date. With interval mapping, this QTL reached the highest LOD 6.36 at 27 dpi (11.6%)

When observations for maturity type were used as trait value in MapQTL, two K* peaks were observed near markers eACAmCAC_19 (K* = 31) and Sti032 (K* = 41) on paternal and maternal linkage group *5*, respectively. On female and male chromosome *5*, the QTL involved in percent infected foliage and maturity type coincided; therefore, we proceeded with QTL analysis using maturity-corrected resistance (MCR) (Bormann et al. 2004; Visker et al. 2004). This trait value represents late blight resistance compensated for the effect of maturity, and was obtained by subtracting maturity values from percent foliage infection, leaving the residuals. MapQTL analysis using the residuals (MCR) resulted in the detection of the same QTL on chromosome *4* with equal significance, but the QTL on chromosome *5* were no longer detected ($P > 0.05$).

In conclusion, among the initial three QTL, we deduced that the large-effect QTL near marker eATGmCAG_235 on chromosome *4* of the resistant parent is a major-effect QTL involved in *Phytophthora* resistance. The smaller-effect QTL, both residing on chromosome *5* of either the susceptible and resistant parent, are QTL involved in plant maturity, and affect late blight infection indirectly.

Qualitative genetic analysis of resistance.

In addition to quantitative trait analysis, an attempt was made to categorize offspring clones in a resistant and susceptible class. The disease progress curves of each offspring genotype are shown in Figure 3. This figure suggests that the most clear-cut classification can be made at 11 dpi. The distribution at 11 dpi is presented in Figure 4. At 11 dpi, the susceptible group had an average foliage infection of 64% whereas the resistant group was on average 7% attacked. On the last day of observation (29 dpi), the susceptible group was 100% infected and the resistant group had an average foliage infection of 88%, using the tentative classification of the offspring at 11 dpi. The 11 dpi classification resulted in a segregation of 150 resistant and 74 susceptible genotypes, which is a significant deviation from a 1:1 ratio ($\chi^2 = 25.8$; $P < 0.0001$). Linkage analysis with this locus representing late blight resistance along with the molecular markers resulted in the construction of a linkage group with a locus named $R_{Pi\text{-}mcd1}$ (Fig. 5). The mapping

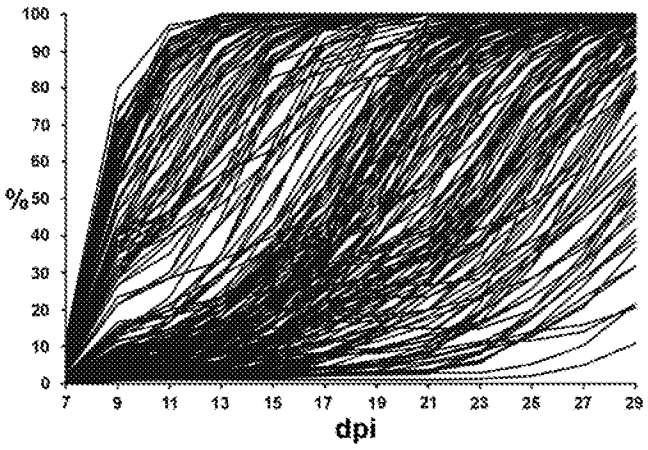


Fig. 3. Late blight disease progress curve in percentage of leaf area infected across 23 days postinfection (dpi) of 244 offspring clones of *Solanum microdontum* BC2 population RH94-076.

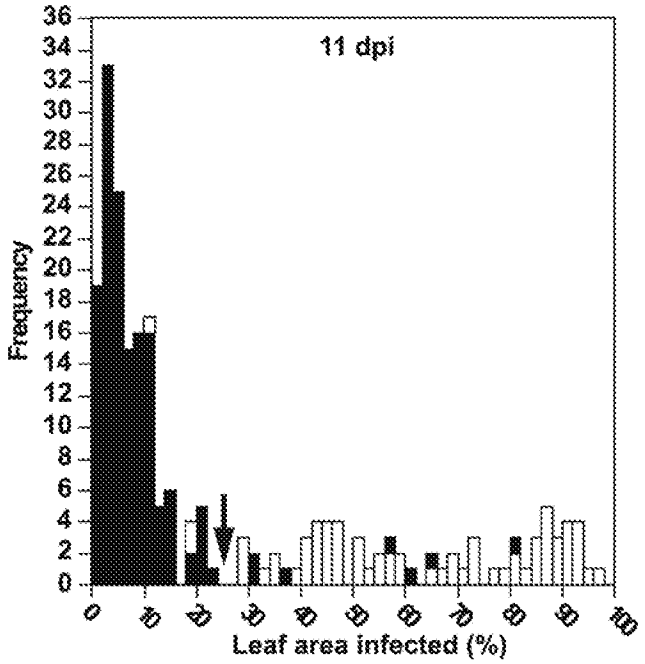


Fig. 4. Histogram showing the frequency distribution of percent leaf area infected by late blight at 11 days postinfection (dpi), the observation date which gives the most clear-cut division between resistance and susceptibility. The arrow indicates the cut-off value (the average population infection at 11 dpi = 25.7%). Genotypes to the left of the arrow were classified as resistant, genotypes to the right were classified as susceptible. Black = $R_{Pi\text{-}mcd1}$ present and white = $R_{Pi\text{-}mcd1}$ not present according to flanking markers.

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results of $R_{Pi\text{-}mcd1}$ allowed validation of the qualitative genetic analysis of late blight resistance, because the expected resistance genotype, based on flanking markers, could be compared with the observed phenotype based on tentative classification. Only three false-resistant phenotypes (clones 59, 187, and 232) and seven false-susceptible phenotypes (clones 20, 21, 84, 135, 140, 264, and 276) among 224 offspring classifications were identified (Fig. 4). All of these false resistant or susceptible classifications belonged to genotypes without recombination event in linkage group *4* and, thus, could not interfere with the position of $R_{Pi\text{-}mcd1}$ on the short arm of chromosome *4*. At 13 dpi, three additional resistant plants were incorrectly scored as susceptible and one susceptible plant was incorrectly scored as resistant.

Based on the absence or presence of $R_{Pi\text{-}mcd1}$ in the offspring, the two average disease progress curves could be calculated. These average curves clearly differ in slope. At 50% loss of leaf tissue, the infection rates were 8.4% (without $R_{Pi\text{-}mcd1}$) and 5.1% (with $R_{Pi\text{-}mcd1}$) increase of foliage infection per day. The average difference in time to reach the level of 50% leaf infection was 11.3 days between genotypes without $R_{Pi\text{-}mcd1}$ compared with genotypes with $R_{Pi\text{-}mcd1}$.

Anchoring of $R_{Pi\text{-}mcd1}$ and saturation of the chromosome *4* linkage map.

The identity of the linkage group comprising the QTL or $R_{Pi\text{-}mcd1}$ locus was first indicated by several AFLP markers which were known chromosome-*4*-specific markers. This was confirmed when RFLP marker TG339, converted into a cleaved amplified polymorphic sequence (CAPS) marker, mapped six recombination events (2.8 cM) below $R_{Pi\text{-}mcd1}$ (toward the centromere) as is shown in Figure 5.

BSA with 256 primer combinations of PstI+NN/MseI+ANN resulted in five bulk-specific bands, of which two were false-positive bands, two markers pCTmAGC_378.0 and pCTmACG_90 mapped in coupling phase, and marker pCTmACG_310, in repulsion phase, showed complete cosegregation with $R_{Pi\text{-}mcd1}$. The second BSA, with 256 primer combinations PstI+NN/MseI+CNN, did not result in additional markers.

Tomato COS marker T0703, converted into a CAPS marker, mapped at the same position as TG339. Tomato RFLP marker CT229, converted into a CAPS CT229, mapped nine recombination events (4.2 cM) distal of $R_{Pi\text{-}mcd1}$. By the addition of this marker, marker pGTmACG_310, no longer co-localized with $R_{Pi\text{-}mcd1}$; a genotype which previously was thought to have a singleton event, proved to be a true recombinant, with the recombination between marker pGTmACG_310 and $R_{Pi\text{-}mcd1}$.

AFLP marker pGTmACG_310 was excised from gel and sequenced, and a Blastn search resulted in strong homology to tomato BAC AF411807 (van der Hoeven et al. 2002), which was assigned to chromosome *4* of tomato between markers CT229 and TG370 (Tanksley et al. 1992). The sequence of marker pGTmACG_310 was aligned with vector NTI to position 95,735 to 95,837 of BAC AF411807, which is close to the R end of this 95,845-bp-long BAC sequence.

The segregation pattern based on the classification using 11 dpi positioned $R_{Pi\text{-}mcd1}$ proximal to marker pGTmACG_310 on the graphical genotype map, with one recombination event between marker pGTmACG_310 and $R_{Pi\text{-}mcd1}$ at a distance of 0.5

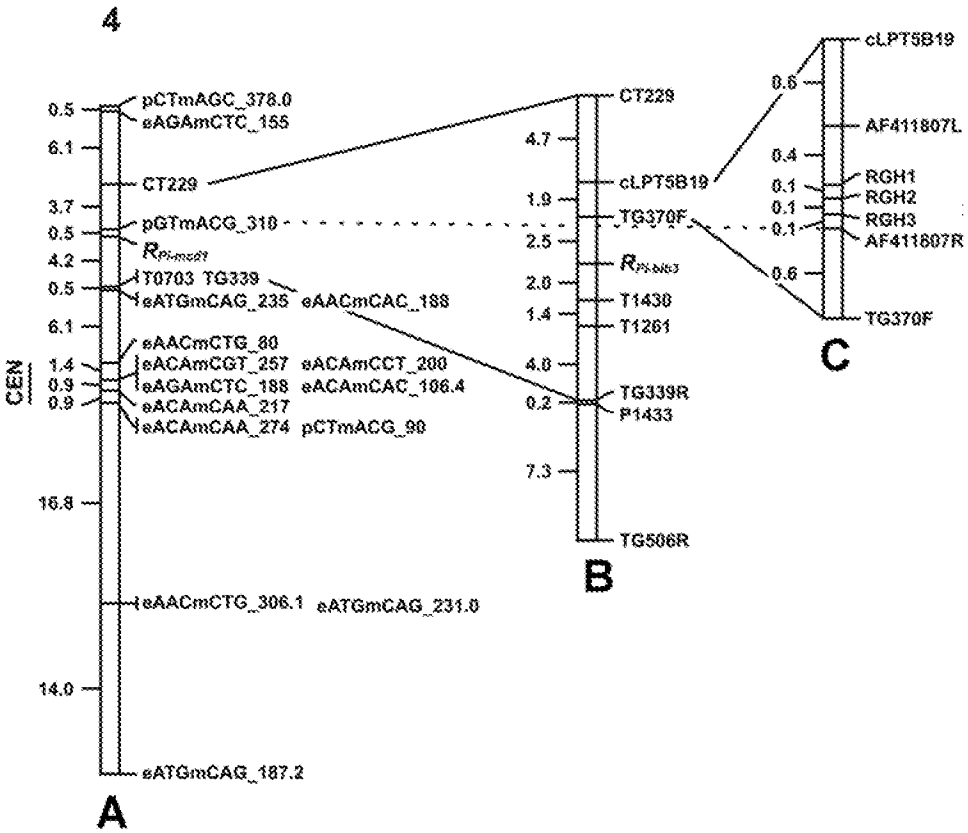


Fig. 5. $R_{Pi\text{-}mcd1}$ maps on chromosome *4* to a known cluster of resistance genes and nucleotide-binding site leucine-rich repeat resistance gene candidates. **A,** Genetic map of chromosome *4* of the maternal resistant parent RH90-038-21 showing the location of $R_{Pi\text{-}mcd1}$. CEN = centromere location. **B,** Genetic map of a part of chromosome *4* with the location of $R_{Pi\text{-}blb3}$ (Park et al. 2005b). **C,** High-resolution map of the chromosome *4* interval distal to $R_{Pi\text{-}blb3}$ with the genetic position of CAPS marker AF411807L and AF411807R based on the BAC end sequences, and three resistance (*R*) gene homologs (RGH1-3) of tomato BAC AF4011807. Amplified fragment length polymorphism marker pGTmACG_310 from this study was identified on BAC AF411807 near the position of AF411807R.

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cM. $R_{Pi\text{-}mcd1}$ was positioned at a distance of nine recombination events (4.2 cM) distal to the flanking markers T0703 and TG339. $R_{Pi\text{-}mcd1}$ appeared to be located on the short arm of chromosome *4*. Centromere location was inferred from the clustering of *Eco*RI/*Mse*I markers around the centromere (Park et al. 2007; van Os et al. 2006) and from tomato and AFLP anchor markers.

Validation of CAPS markers.

CAPS markers TG339, CT229, and T0703 were tested for *S. microdontum*-specific polymorphism in a range of germplasm (n = 96) comprising DNA of *S. tuberosum, S. berthaultii, S. microdontum, S. bulbocastanum, S. tarijense, S. fendleri, S. hougassi*, and *S. vernei*. None of the marker alleles were exclusively diagnostic for $R_{Pi\text{-}mcd1}$. Neither were these markers specific for wild germplasm and absent in cultivated potato. Therefore, these markers, in combinations with these restriction enzymes, which work well for this mapping population, cannot be used for marker-assisted breeding in a wider range of germplasm.

DISCUSSION

In this article, a range of *Phytophthora* assays has been used that are usually not applied in combination on the same material. Although the in vitro and detached-leaf assays did not contribute to the localization of $R_{Pi\text{-}mcd1}$, the joint results allowed us to characterize the phenotype of $R_{Pi\text{-}mcd1}$ as a locus involved in late blight resistance, and to evaluate our results in relation to current models on late blight resistance.

The in vitro assay resulted in inconclusive data, because most of the genotypes of the *S. microdontum* mapping population were sporulating within 7 days although half of the population initially showed a weak HR. With the detached-leaf assay, clones of the mapping population showed inconsistent phenotypes. These results indicated that some form of resistance should be present in this material; however, a qualitative genetic interpretation of the in the in vitro and detached-leaf assays was not obvious. For a quantitative interpretation, data were subsequently collected from a field assay. When using rAUDPC and percent foliage infection as trait values, three QTL were detected. One major QTL associated with resistance was located on chromosome *4*. The two other QTL were detected on the maternal and paternal linkage groups corresponding to potato chromosome *5*. However, when the trait values were corrected for maturity, QTL on chromosome *5* could no longer be detected. This indicates that the QTL of chromosome *5* were involved in plant maturity.

The rAUDPC is currently the most accepted trait value to assess resistance levels. In this study however, the best trait value for QTL analysis proved not to be the rAUDPC but percent foliage infection at 9 to 11 dpi. The latter method showed the highest association in K* values in QTL analysis. The explanation for the lower power of rAUDPC as a trait value in QTL identification can be the effect of inclusion of too late and, thus, indiscriminative observations within the highly informative observation at the beginning.

However, the best approach to localize hereditary factors is not QTL analysis at all but Mendelian classification. Classification of the foliage infection values resulted in accurate localization of $R_{Pi\text{-}mcd1}$ on chromosome *4*. At this genetic position, only 10 false positives or false negatives were inferred using markers flanking $R_{Pi\text{-}mcd1}$ among 224 offspring. Timing is important, because the observations at 7 to 11 dpi—the early start of infection—resulted in a reliable classification. When later time points were used, additional genotypes resulted in misclassification (based on the flanking markers) as resistant or susceptible. Classification results in a single genetic position, whereas the QTL peak (Sandbrink et al. 2000) occupies a much larger genetic interval. It cannot be excluded that the resistance QTL on chromosome *4* reported by Sandbrink and associates (2000) is identical to the locus mapped in this study, although a different accession, BGRC 24981, was used.

Marker saturation with BSA resulted in the identification of marker pGTmACG_310 at a distance of 0.4 cM of $R_{Pi\text{-}mcd1}$. The DNA sequence of this marker showed strong homology to a part of tomato BAC AF411807, which had been identified in a previous study (Park et al. 2005a). Park and associates identified the same BAC clone with AFLP marker pATmAGA_307 which was tightly linked (<0.1 cM) to another late blight resistance gene, $R_{Pi\text{-}abpt}$, on chromosome *4*. On this BAC clone, three nucleotide-binding site leucine-rich repeat (NBS-LRR) genes were identified with high homology to AFLP marker pATmAGA_307, which also has an NBS-LRR sequence. Currently, three more resistance genes, *R2*, $R_{2\text{-}like}$, and $R_{Pi\text{-}blb3}$, have been mapped along with $R_{Pi\text{-}abpt}$ in this chromosome *4* *R*-gene cluster (Li et al. 1998; Park et al. 2005a, b, and c). Our data indicate that $R_{Pi\text{-}mcd1}$ is identified as the fifth member of this *R*-gene cluster.

Strong indication that $R_{Pi\text{-}mcd1}$ belongs to the family of NBS-LRR genes.

In our view, the mapping of $R_{Pi\text{-}mcd1}$ in a well-known *R*-gene cluster of NBS-LRR genes is the most fascinating result of this study. Theoretically, we cannot exclude that the gene underlying the $R_{Pi\text{-}mcd1}$ locus does not belong to the family of NBS-LRR genes. However, other projects in our lab (E. van der Vossen, *personal communication*) suggest a limited genetic and physical size of the region on potato chromosome *4*, comprising the series of NBS-LRR genes underlying the $R_{Pi\text{-}blb3}$, *R2*, $R_{2\text{-}like}$, and $R_{Pi\text{-}abpt}$ loci. Thus, it appears that it is more plausible to accept the hypothesis that $R_{Pi\text{-}mcd1}$ is a member of an allelic series of *R* genes than to expect an entirely different kind of gene, although supporting information is required to arrive at a final conclusion.

Embarking from the hypothesis that $R_{Pi\text{-}mcd1}$ is an NBS-LRR gene, we should now discuss the phenotypic effects observed for $R_{Pi\text{-}mcd1}$ in comparison with the effects associated with NBS-LRR-based *R* genes involved in late blight resistance. To this end, we first need to give an overview of the different models of the potato–late blight interaction, as they have appeared in the scientific literature (Table 1).

Different models of potato–late blight interaction.

The most intriguing distinction between late blight resistance models is based on *R*-gene-based and non-*R*-gene-based resistance. This distinction is of primary importance because, in general, *R*-gene-based resistance is assumed to be race specific and not durable, whereas non-*R*-gene-based resistance should be durable, race-nonspecific, and, thus, valuable (van der Plank 1968). The *R*-gene-based resistance is based on monogenic factors. The first resistance model in Table 1, "non-durable race-specific resistance," refers to the *S. demissum*-derived *R* genes which were soon defeated once cultivars were grown on a larger scale. Although lumped in one model, there are differences between the effects of these *R* genes. Some *R* genes are known as stronger-effect (*R1* and *R3*) or weaker-effect (*R10*) *R* genes. Furthermore, although defeated, *S. demissum R* genes such as *R2* still offer protection in certain growing areas (Pilet et al. 2005), which might be explained by the possibility that a proportion of the *Phytophthora* population participating in the epidemic carried the avirulence factor to *R2*, although increased deployment of cultivars with *R2* is unlikely to make a lasting contribution to late-blight control.

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The second resistance model, "broad-spectrum resistance," refers to *S. bulbocastanum*-derived resistance (Song et al. 2003; van der Vossen et al. 2003, 2005). Because this resistance is based on the usual NBS-LRR-type *R* gene, we assume that broad spectrum only indicates the current absence of a compatible race. There is no reason to assume that the durability of these newly deployed *R* genes would differ from the durability experienced in the 20th century with *S. demissum*-derived resistance. Therefore, we anticipate that our first and second models are not essentially different.

The third model, "residual resistance," refers to the contribution of defeated *R* genes to the quantitative level of field resistance (Durel et al. 2003; Pedersen and Leath 1988; Stewart et al. 2003). A classical example of a residual effect of defeated *R* genes has been presented for various *Xanthomonas* resistance genes in rice (Li et al. 1999). This term should not be confused with the horizontal resistance observed after vertical resistance has been defeated (van der Plank 1968). Here, the residual effect is ascribed to the defeated *R* gene itself (Durel et al. 2003). Therefore, residual resistance should not be regarded as a different model but as an asset of the normal *R* genes belonging to the first model.

The fourth model, "field resistance in *R*-gene-free cultivars," was historically pursued in response to the lack of durable *R* genes. Using old potato cultivars, Colon and associates (1995) observed that current resistance ratings correlated well with ratings given between 1929 and 1954. This suggests that old potato cultivars, free from *R* genes, offer a stable and heritable source for resistance. Although Colon and associates (1995) noticed the correlation between plant maturity and late blight resistance, they suggested the presence of durable resistance, acting in addition to the maturity effect (Toxopeus 1958). The stability of resistance to *P. infestans* in old cultivars without *R* genes (Colon et al. 1995; van der Plank 1971) resulted in a new breeding strategy as promoted by Turkensteen (1993) and Landeo and associates (1995). However, stable field resistance as perceived by Van der Plank and Colon and associates in old potato cultivars is, in our opinion, nothing but the effect of plant maturity and, perhaps, the residual effect of indigenous *S. tuberosum R* genes. This view was also strongly advocated by Allefs and associates (2005).

The fifth model, "QTL involved in resistance," is represented by a number of studies that were among the first to fully exploit the molecular genetic tools and QTL analysis. These studies embarked, in general, on the assumptions that resistance is phenotypically quantitative, race nonspecific, and possibly polygenically inherited. The choice of the plant material in these studies was largely influenced by the desire to reidentify not *R* genes but loci involved in partial, field, or horizontal resistance. The major QTL identified by the studies of Leonards-Schippers and associates (1994), Collins and associates (1999), and Oberhagemann and associates (1999) were always on potato chromosome *5* and coincided with a QTL for maturity type. In our opinion, the major QTL involved in resistance are essentially the same as the loci involved in field resistance of the fourth model (van Eck and Jacobsen 1996). Current methods of data analysis include maturity as co-variable during QTL analysis. Alternatively, resistance data are corrected for plant maturity (MCR), hence leaving the residual variance as trait value (Bormann et al. 2004; Visker et al. 2003). In view of our remarks on the fallacy of field resistance in *R*-gene-free material, we assume that MCR is caused by genes that belong to the NBS-LRR family.

Sifting through these views on the potato–late blight interaction, we construe that, in essence, resistance is determined by two factors only: plant maturity and *R* genes that belong to the NBS-LRR family. The latter factor can be subdivided in stronger- and weaker-effect *R* genes, broad-spectrum and race-specific *R* genes, broken *R* genes with larger or smaller residual effects, and so on. Nevertheless, all these classes are to be ascribed to NBS-LRR genes and the underlying elicitor–receptor model (Allefs et al. 2005; Kamoun et al. 1999).

Evaluation of $R_{Pi\text{-}mcd1}$ in relation to current models of potato–late blight interaction.

Having described i) the phenotypic effects of $R_{Pi\text{-}mcd1}$ in different assays, ii) the quantitative and qualitative genetic analysis of the resistance, and iii) its genetic position in an *R*-gene cluster on potato chromosome *4*, we now wish to evaluate $R_{Pi\text{-}mcd1}$ in relation to the models of the potato–late blight interaction described above.

Our quantitative genetic analysis of $R_{Pi\text{-}mcd1}$ resulted in explaining 50% of the phenotypic variance. This suggests partial resistance, as described in our fourth and fifth models; however, our phenotypic observations disagree with the commonly held views on partial resistance. By definition, partial resistance results in a reduced epidemic build-up of the pathogen, notwithstanding a susceptible, nonhypersensitive infection type (Parlevliet and van Ommeren 1975). Partial resistance due to genes with small effects is thought not to be based on receptor-elicitor recognition (McDonald and Linde 2002). The effect of $R_{Pi\text{-}mcd1}$ is associated with an HR, followed by sporulation. The HR is not in accordance with the definition of partial resistance but fits more closely with observations described by Vleeshouwers and associates (2000), where the quantitative nature of *P. infestans* resistance is explained by differences in timing of HR induction and differences in percentage of cells displaying HR after infection, possibly due to inadequate or delayed recognition of elicitors by weak-effect *R* genes. Their histological studies also indicated that, at the cellular level, the difference between compatible and incompatible interactions was quantitative rather than qualitative in nature. This may result in phenotypes which have been described by terms such as restricted lesion, a restricted but sporulating lesion, or a

Table 1. Overview of various models used in literature to describe the potato–late blight interaction in qualitative and quantitative genetic terms

Model	Genes	Literature reference
R-gene-based resistance[a]		
1. Nondurable race-specific resistance	*R1-R11*	Black et al. 1953; Eide et al. 1959; Malcolmson 1969; Malcolmson and Black 1966
2. Broad-spectrum resistance[b]	*RB*, $R_{Pi\text{-}blb1}$	Song et al. 2003; van der Vossen et al. 2003
3. Residual resistance	*R1*, *R10*, *R11*	Stewart et al. 2003
QTL-based resistance[c]		
4. Field resistance in *R*-gene-free cultivars	...	Colon et al. 1995; Landeo et al. 1995; Turkensteen 1993
5. QTLs involved in resistance[d]	Various QTLs	Bormann et al. 2004; Collins et al. 1999; Leonards-Schippers et al. 1994; Oberhagemann et al. 1999; Visker et al. 2003

[a] Where often monogenic *R*-gene based resistance is implied.
[b] Race-nonspecific resistance.
[c] QTL = quantitative trait locus; where often a polygenic and non-*R*-gene based resistance is implied.
[d] With or without correction for plant maturity.

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spreading and sporulating lesion. In particular, the second table of Vleeshouwers and associates (2000) demonstrates that HR is observed during all forms of potato–late blight interaction (susceptible, *R*-gene-based, and nonhost resistance). Hence, HR and successful infection are not a paradox.

Our *S. microdontum*-derived BC2 mapping population was susceptible in the in vitro assay, susceptible in the detached-leaf assay, and showed a delayed infection in a field assay. The in vitro assay is known to be very efficient but also very rigorous and will allow the identification only of *R* genes with strong effects (i.e., which always show full resistance to avirulent *P. infestans* isolates) (Huang et al. 2005). Likewise, the detached-leaf assay is also more suitable for *R* genes with strong effects (Vleeshouwers et al. 1999). From this perspective, $R_{Pi\text{-}mcd1}$ should be considered as an *R* gene with a weak effect. Thus, the phenotype of $R_{Pi\text{-}mcd1}$ resembles the incompatible reaction of the *S. demissum* differentials *R10* and *R11* (Huang et al. 2005), supporting our conclusion that $R_{Pi\text{-}mcd1}$ may belong to the family of NBS-LRR genes.

We have never observed any race-specific interaction for $R_{Pi\text{-}mcd1}$. The 1- to 2-week delay in infection in the field has been observed consistently in material from various backcross generations and regardless of the late blight races (*unpublished results*). The material is also used by commercial breeders, who annually confirm the consistent effect of delay of infection against randomly blown-in *P. infestans* isolates. Therefore, $R_{Pi\text{-}mcd1}$ cannot be regarded as a broken *R* gene (first model). Neither can $R_{Pi\text{-}mcd1}$ be regarded as a gene involved in residual resistance (third model) because, from a historical perspective, the $R_{Pi\text{-}mcd1}$ locus never conferred complete resistance (was never unbroken) and all races ultimately sporulate (there is no distinction between compatible and incompatible races).

This effect results in the conclusion that $R_{Pi\text{-}mcd1}$ combines two features. It is both a weak resistance gene as well as a broad-spectrum (race-nonspecific) resistance gene. Nevertheless, the value of a weak *R* gene in economic terms is considerable. When infection is delayed for 1 week in organic potato cultivation, at a biomass production of 1 ton per hectare per day, the economic value of such a delay exceeds $1,500 per hectare.

For decades, it has been advocated that breeding for durable late blight resistance should require the screening of potato germplasm for the absence of *R* genes. These nondurable *R* genes only blurred the view on the level of partial, race-nonspecific, field resistance. in our opinion, this is a flawed strategy and may have unnecessarily removed *R* genes from the potato gene pool. Moreover, it ignores the possible residual effect of broken *R* genes, as was demonstrated by Stewart and associates (2003). The $R_{Pi\text{-}mcd1}$ gene described in this article indicates that partial resistance could well be *R*-gene based. We conclude that NBS-LRR-genes, sometimes in black and white but also in many shades of gray, all posses value in breeding for late blight resistance.

This example of $R_{Pi\text{-}mcd1}$ illustrates the unwarranted relation between phenotype, experimental approach, and scientific model. Nonabsolute phenotypic differences should not necessarily be addressed by a quantitative genetic approach. Quantitative genetic studies should not necessarily result in QTL and exclude *R* genes from the hypothesis tested. In our opinion, the distinction that *R*-gene-based resistance should always give absolute resistance and that partial resistance is always mediated by QTL should be reevaluated.

MATERIALS AND METHODS

Plant material.

A diploid interspecific mapping population RH94-076 (n = 224) was created with a cross between RH90-038-21 and RH88-025-50. The pedigree of RH94-076 is presented in Figure 1. Because of the susceptible back-cross (BC) parents and the agronomical selection in the BC1 offspring, this BC2 mapping population should be regarded as essentially a susceptible *S. tuberosum* genetic background with less than 12.5% *S. microdontum* introgression. The *S. microdontum* accession BGRC 18302 (CGN21342) was collected by Hjerting, during a Danish expedition (HPR 0293) in 1965 and 1966 to Argentina.

In vitro assay.

In all, 371 newly sown genotypes of RH94-076 were tested in an in vitro assay as described by Huang and associates (2005) with *P. infestans* isolate 90128 (race 1, 3, 4, 6, 7, 8, 10, and 11) provided by F. Govers, Laboratory of Phytopathology, Wageningen University, The Netherlands. Symptoms were recorded at 3, 4, 5, 6, and 7 days after infection. Sporulation or large-scale necrosis was interpreted as susceptible. An HR or lack of symptoms was interpreted as resistant. The susceptible cv. Bintje and the $R_{Pi\text{-}abpt}$ containing resistant genotype 707TG11-1 (Park et al. 2005a) were used as control plants.

Detached-leaf assay.

Leaves collected from greenhouse plants of 96 genotypes of RH94-076 were tested in four replications with a detached-leaf assay (Vleeshouwers et al. 1999). Parents RH90-038-21 and RH88-025-50 were included as control plants. Complex isolate 90128 (race 1, 3, 4, 6, 7, 8, 10, and 11) was used to determine leaf resistance. Inoculation was performed as described by Vleeshouwers and associates (1999), except that the concentration of the inoculum was adjusted to 5×10^5 zoospores/ml.

Field test 2001.

The field test was performed in 2001 on 224 genotypes of RH94-076. The location of the field was the Meenthoeve, in the area near Wageningen, The Netherlands, on sandy soil. Tubers were planted on 15 May 2001. Experimental design was a randomized block design. Four tubers per genotype were planted. The four plants per genotype were treated as one experimental unit. Inoculation was performed with a spore suspension of a complex *P. infestans* isolate IPO-82001 (race 1, 2, 3, 4, 5, 6, 7, 10, and 11; provided by W. Flier, Plant Research International) as described by Vleeshouwers and associates (1999) on 11 July. Because of a lack of infection, the field was reinoculated with the same isolate on 17 July. Infection levels were scored 12 times, every other day, over a period of 3.5 weeks as the average leaf area covered by lesions (as a percentage of the total leaf area). Observation time points are indicated as days postinfection, taking 17 July as day zero. Values of rAUDPC were calculated as described in Fry (1978).

Maturity type was scored on a fungicide-protected field experiment in the third week of August on an ordinal scale ranging from 2 (green and vigorous, late to very late) to 6 (yellowing and plant is sagging) to 10 (dead, very early plant types).

Classification of foliage infection values into resistant or susceptible phenotypes.

Field test observations from 2001 were converted into a tentative phenotypic classification of descendants with or without resistance factors, taking the observations at 7 to 13 dpi. This classification used the population mean as threshold. Offspring with infection lower or higher than the population mean were classified as resistant or susceptible, respectively.

DNA extraction.

Genomic DNA of the individuals of mapping population RH94-076 and parents RH90-038-21 and RH88-025-50 was isolated from young leaf material as described by Van der

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Beek and associates (1992). Tissue was ground with steel balls using a Retsch machine (Retsch Inc., Haan, Germany) in the STE buffer as described, with volumes adapted to 2-ml deep 96-wells Costar plates (Corning Inc., Corning, NY, U.S.A.).

Amplified fragment length polymorphism analysis.

AFLP analysis was performed according to Vos and associates (1995). Twelve primer combinations were used: E+AAC/M+CAC, E+AAC/M+CAG, E+AAC/M+CCA, E+AAC/M+CTG, E+AAT/M+CGA, E+ACA/M+CAA, E+ACA/M+CAC, E+ACA/M+CCT, E+ACA/M+CGT, E+AGA/M+CTC, E+ATG/M+CAG, and E+ATG/M+CTC. AFLP fragments were visualized on radioactive gels and scored visually for absence or presence. AFLP marker names are composed of the restriction enzyme combination followed by the three selective nucleotides of the primers followed by the migration on gel relative to the 10-base ladder (Sequamark, Research Genetics, Huntsville, AL, U.S.A.). Marker names with sizes in tenths of nucleotides refer to markers mapped before in reference populations. These reference markers can be used to assign linkage groups to potato chromosomes. The other markers are unique for this material.

Map construction.

With JoinMap (van Ooijen and Voorrips 2001), 210 AFLP markers were assigned to linkage groups using a LOD threshold of 5.0 for grouping. In a second version of the map, 32 simple-sequence repeat (SSR) markers were included for confirmation of chromosome identity. The entire map was composed of 12 maternal and 12 paternal linkage groups, with 26 markers remaining unassigned. Details on the map and map construction will be published elsewhere (J. Werij, *unpublished data*). The tentative classification of late blight resistance phenotypes, described above, was used as marker data of the $R_{Pi\text{-}mcd1}$ locus and included in map construction.

Bulked segregant analysis.

Bulked segregant analysis (BSA) (Michelmore et al. 1991) was applied to identify additional markers in the proximity of the locus involved in resistance, with the aim to further saturate the genetic map. Four bulks were composed as follows: Br and Bs, each containing eight nonrecombinant genotypes with a resistant or susceptible phenotype, respectively; and two additional bulks, Brc (n = 5) and Bsc (n = 8), composed to narrow down the target window on chromosome *4*, using descendants with crossover events between markers flanking the locus involved in resistance. The crossover events were inferred between AFLP markers eAGAmCTC_155 and eATGmCAG_187.2 with a distal position, as known from our marker catalog. Bulks were analyzed with 256 PstI+NN/MseI+ANN and 256 PstI+NN/MseI+CNN primer combinations. Fluorescently labeled AFLP fragments were visualized on a denaturing polyacrylamide gel using a NEN Global Edition IR2 DNA Analyzer (LI-COR Biosciences, Lincoln, NE, U.S.A.). AFLP primer combinations using *Pst*I/*Mse*I template were used, because the chromosomal distribution of *Pst*I/*Mse*I compares favorably with *Eco*RI/*Mse*I-derived AFLP markers. The majority of *Eco*RI/*Mse*I-derived AFLPs cluster in centromeric regions as shown by Van Os and associates (2006).

Excision of AFLP fragments.

*Pst*I primer PstI+GT was radioactively labeled with ^{33}P. AFLP was performed according to (Vos et al. 1995). AFLP bands of marker pGTmACG _310 of eight samples (two of RH90-038-21, two of R88-250-50, plus four susceptible individuals) were excised from gel and dissolved in 50 µl of water. Supernatant was reamplified with P+0 and M+A primers and purified with a G50 column before sequencing. pGTmACG_310 sequence was Blastn.

CAPS markers.

Further anchoring of the resistance locus was achieved with chromosome *4* specific markers TG339, CT229, and T0703. Polymerase chain reaction (PCR) primers were designed using sequence information from the SOL Genomics Network with Primer3 software (Rozen and Skaletsky 2000). PCR products were digested with 27 restriction enzymes to identify polymorphic sites. The potato version of TG339 was developed using GenBank accession number X87370 (*S. tuberosum* mRNA for 14-3-3 protein) obtained with Blastn using the original tomato TG339 restriction fragment length polymorphism (RFLP) sequence. TG339 primers were F:GCTGAACGCTATGAGGAG ATG and R:TGAGGTTATCACGCAGAAGTTG. The CAPS polymorphism was obtained by digestion with *Mnl*I. Marker CT229 was amplified with primers F:TTGTGAGTGGTGAA CTACGGGC and R:CGGCAATGGTTATGGGAACG (Park et al. 2005b), and displayed a CAPS polymorphism using restriction enzyme *Hpy*CH4IV. The sequence of tomato COS marker T0703 was used for conversion into a CAPS marker. Primers for the T0703 marker were F:CCAGTAAGAACAAGCCG ATT and R:ATCACCAATTACGCGATCTA. A polymorphism was obtained upon restriction with *Bme*1390I.

Chromosome *4* map construction.

The marker order of maternal linkage group *4* including, the added AFLP markers from BSA and CAPS markers TG339, CT229, and T0703, was reassessed with RECORD (van Os et al. 2005) and visually inspected by graphical genotyping the raw data in MS-Excel using the conditional formatting of cells conditional to marker genotype and linkage phase. Marker pairs without obvious recombination events were placed at the same map position. This avoids suggestive marker orders without clear data support. Final map length of the linkage group, including $R_{Pi\text{-}mcd1}$, reflects the sum of adjacent recombination frequencies (Stam 1993).

QTL analysis.

QTL analysis was performed with MapQTL 5 (van Ooijen 2004) using a linkage map of 1,179.2 centimorgans (cM) (maternal plus paternal map length). An initial search for QTL was performed with Kruskal-Wallis because no assumptions of normality were made. For interval mapping purposes, the average leaf area covered by lesions (percent) per observation and per genotype was converted to degrees by the variance-stabilizing angular transformation (angle whose sine is the square root of the percentage of foliage infection).

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AUTHOR-RECOMMENDED INTERNET RESOURCES

National Center for Biotechnology Information Blast server: www.ncbi.nl.nih.gov/BLAST
SOL Genomics Network website: www.sgn.cornell.edu
Wageningen UR Plant Breeding website: www.dpw.wau.nl
The Wageningen UR Plant Breeding group Ultra dense recombination map of potato website: www.plantbreeding.wur.nl/potatomap

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ORIGINAL PAPER

GpaXI$^{l}_{tar}$ originating from *Solanum tarijense* is a major resistance locus to *Globodera pallida* and is localised on chromosome *11* of potato

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Abstract Resistance to *Globodera pallida* Rookmaker (Pa3), originating from wild species *Solanum tarijense* was identified by QTL analysis and can be largely ascribed to one major QTL. *GpaXI*$^{l}_{tar}$ explained 81.3% of the phenotypic variance in the disease test. *GpaXI*$^{l}_{tar}$ is mapped to the long arm of chromosome *11*. Another minor QTL explained 5.3% of the phenotypic variance and mapped to the long arm of chromosome *9*. Clones containing both QTL showed no lower cyst counts than clones with only *GpaXI*$^{l}_{tar}$. After Mendelising the phenotypic data, *GpaXI*$^{l}_{tar}$ could be more precisely mapped near markers GP163 and FEN427, thus anchoring *GpaXI*$^{l}_{tar}$ to a region with a known *R*-gene cluster containing virus and nematode resistance genes.

Introduction

The white potato cyst nematode (PCN) *Globodera pallida* (Stone) is a major pest in potato. Early work on resistance breeding has been concentrated on the golden potato cyst nematode *G. rostochiensis*, although the distinction between *G. rostochiensis* and *G. pallida* has been introduced only since 1974 (Stone 1972). Breeders succeeded in producing potato cultivars resistant to *G. rostochiensis*, predominantly by incorporating the *H1* gene from *S. tuberosum* ssp. *andigena* CPC 1673 (Ellenby 1952; Huijsman 1957; Ross 1979). Since the discovery of *H1*, many more resistance genes against *G. rostochiensis* have been identified (reviewed by Gebhardt and Valkonen 2001). Due to the extensive use of *G. rostochiensis* resistant cultivars, *G. pallida* populations, free of competition from *G. rostochiensis*, have become the majority of the cyst nematode pest population in Europe. Great emphasis is now placed on producing varieties with resistance against a broad spectrum of populations (Hancock 1996; Turner 1989). Breeding for resistance to *G. pallida* was initially hampered by the seemingly quantitative inheritance of the resistances first identified in *S. vernei* and *S. tuberosum* spp. *andigena* (Dale and Phillips 1982; Ross 1986). The resistance to pathotypes Pa2 and Pa3 from *S. tuberosum* ssp. *andigena* CPC 2802 was first thought to be monogenic (the *H3* gene) (Howard et al. 1970), but was later proven to be polygenic (Dale and Phillips 1982). Genetic analysis of a SCRI progenitor clone with resistance against *G. pallida*, and descending from CPC 2802, resulted in the identification of a major QTL on linkage group *4* (probably the *H3* gene), as well as a minor QTL on linkage group *11* (Bryan et al. 2004). The higher diversity of the *G. pallida* populations compared to *G. rostochiensis* populations also hampered incorporation of effective resistance (Folkertsma 1994; Schnick et al. 1990).

Monogenic resistance to *G. pallida* has been identified. Some appear to be only effective against specific *G. pallida* populations, such as the *H2* locus from

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S. multidissectum, conferring resistance to *G. pallida* populations of pathotype Pa1 (Dunnett 1961), and the *Gpa2* gene. *Gpa2*, derived from *S. tuberosum* spp. *andigena* CPC 1673 confers resistance to a small, distinct population of *G. pallida* Pa2. *Gpa2* maps on chromosome *12* of potato in a single cluster of virus and nematode resistance genes and has been cloned. The protein encoded by *Gpa2* turned out to be of the class of LZ–NBS–LRR type plant resistance genes (Arntzen et al. 1994; Rouppe van der Voort et al. 1999; van der Vossen et al. 2000).

Rouppe van der Voort et al. (2000) identified two QTL that are likely derived from *S. vernei*. One of the two loci, *Gpa5*, explained 61% of the total variation and the resistance conferred by this locus appears to work against a range of *Globodera* populations. The other locus, *Gpa6*, explained 24% of the total variation and acts in a pathotype-specific way. Interestingly, both QTL combined give additive resistance levels. The same holds true for potato clones carrying both QTL $GpaV^{s}_{spl}$ and $GpaXI^{s}_{spl}$ (Caromel et al. 2005). These *S. sparsipilum* derived QTL were mapped on chromosome *5* and *11*, respectively. When combined they do not only show an additive effect on sex ratio of *G. pallida*, but also give a necrotic reaction in roots infected by nematodes; an effect not seen with the individual QTL. Another locus, *Grp1*, with resistance against both PCN species (Rouppe van der Voort et al. 1998a) showed resistance to *G. rostochiensis* line Ro_5-22 and *G. pallida* populations Pa_2-D383 and Pa_3-Rookmaker. *Grp1* was mapped on chromosome *5* in the same region as *Gpa* and *Gpa5*. However, it has not been described in literature that any of the wild *Solanum* sources used to introgress *Grp1* showed resistance against both *G. rostochiensis* and *G. pallida*. It is not excluded that the combined resistance against *G. rostochiensis* and *G. pallida* of *Grp1* is caused by two, or possibly three, tightly linked *R*-genes.

For most *G. pallida* resistance loci described above, the phenotypic segregation data indicated quantitative inheritance. DNA markers offered the potential to trace the Mendelian loci underlying quantitatively inherited nematode resistance and in many cases the resistance appeared to be controlled by single major genes explaining a large proportion of the phenotypic variance. Moreover, the fact that most PCN resistances have been mapped in well-known resistance clusters indicates the likelihood that these major loci are NBS-LRR genes and operate on a gene-for-gene basis (Gebhardt and Valkonen 2001).

In this study, we report a major-effect locus involved in resistance against *G. pallida* Pa3-Rookmaker originating from *S. tarijense*. Qualitative interpretation of the resistance data allowed mapping of the major-effect locus to a known cluster of resistance genes on the long arm of chromosome *11*.

Materials and methods

Plant material

The diploid BC1 mapping population RHAM-061 was obtained from a cross of resistant parent RH90-011-4 and susceptible *S. tuberosum* parent RH89-039-16, as shown in Fig. 1. RH90-011-4 was obtained from an interspecific cross between *S. tarijense* (BGRC 24717/CGN 18107) and MON-46, a dihaploid clone of the susceptible cultivar Mondial.

Nematode resistance assays

A closed container test (Phillips et al. 1980) with *G. rostochiensis* populations ASCRI (Ro1), C262 (Ro2_3), G 1510 (Ro5) was performed on the *S. tarijense* parent RH90-011-4. After 8 weeks in the dark at 18°C, the newly formed cysts that were visible from the outside were counted.

All further *G. pallida* resistance assays were arranged in randomised complete block designs, including parents. As much as 188 offspring genotypes of RHAM-061 were multiplied in vitro and transplanted in 500-ml clay pots to the greenhouse in 3 replicates. Eight replications of the parents as well as the susceptible standard Bintje were included. *G. pallida* population Rookmaker (Pa3), originally sampled from a heavily infested site in Valthe, the Netherlands, was used for the inoculation. Population Rookmaker is one of the most virulent *G. pallida* populations in the Netherlands (Bakker et al. 1992). Four weeks after transplanting, the plants were inoculated. Inoculum of nematodes was prepared as described by Rouppe van der Voort et al. (1997a). Cysts were soaked overnight in tap water before crushing to obtain eggs and second-stage juveniles (J2). The egg/J2 suspension was then sieved through a 100-μm sieve to remove cell walls and debris. The suspension was inoculated to the plants to a final density of 6 eggs/J2 per ml soil. Two and a half months after inoculation, the cysts were elutriated with a Fenwick can and counted (Fenwick 1940).

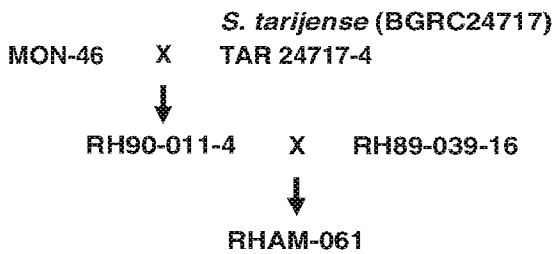


Fig. 1 Pedigree of the interspecific $GpaXI^{l}_{tar}$ mapping population RHAM-061

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DNA extraction

Genomic DNA was isolated from young leaf material as described by van der Beek et al. (1992). Tissue was ground with steel balls using a Retsch machine (Retsch Inc., Haan, Germany) in the STE-buffer, with volumes adapted to 2-ml deep 96-wells Costar plates (Corning Inc., Corning, NY, USA). DNA was visualised on agarose gel to check the integrity.

*Eco*RI/*Mse*I markers

AFLP was performed according to (Vos et al. 1995). Fourteen *Eco*RI/*Mse*I primer-combinations eAACmCCA, eAACmCCT, eAACmCGA, eAACmCTG, eAAGmCCT, eAAGmCGA, eACAmCAA, eACAmCCA, eACAmCTA, eACTmCAA, eACTmCAG, eAGAmCAG, eAGAmCAT and eAGAmCTG were used. AFLP fragments were visualised on radioactive gels and scored visually for absence/presence.

PCR markers

Two chromosome *11* specific markers, GP163 (Brigneti et al. 1997) and FEN427, were used. CAPS marker GP163 is a previously converted RFLP marker, previously used to map potato virus Y resistance gene Ry_{sto} on chromosome *11* (Brigneti et al. 1997). CAPS marker FEN427 was developed on the basis of AFLP marker pATmCAC_427 which was previously used to map $R_{Mc1-fen}$ on chromosome *11* (Draaistra 2006). AFLP band pATmCAC_427 was excised from gel and dissolved in 50 µl water. Supernatant was re-amplified with P + 0 and M + C primers and purified with a G50 column before sequencing. PCR primers for FEN427 were designed on the sequence of AFLP marker pATmCAC_427 with Primer3 software (Rozen and Skaletsky 2000). PCR-products of GP163 and FEN427 were digested with 27 restriction enzymes to identify polymorphic sites (Brugmans et al. 2003).

Map construction

The grouping of markers in linkage groups and the marker order were calculated using the software package JoinMap (Stam 1993). Only AFLP markers with LOD scores >3.0 were included in mapping. Initially the maternal and paternal linkage groups were constructed separately, by taking only the 1:1 segregating AFLP loci (Aa × aa and aa × Aa for the maternal and paternal maps, respectively). The paternal linkage groups could be assigned to potato chromosomes using a reference mapping population that has the susceptible parent RH89-039-16 in common. This reference map SHxRH (Rouppe van der Voort et al. 1997a; van Os et al. 2006) was aligned along with other maps, and resulted in an online catalogue of AFLP markers covering the potato genome (Rouppe van der Voort et al. 1998b). Once the chromosome numbers were known of the paternal linkage groups, subsequently the maternal linkage groups could be aligned using the so called 'bridge markers'. Bridge markers are a small subset of the segregating AFLP loci, which are heterozygous in both parents (Aa × Aa and thus segregating in a 3:1 ratio).

The maternal linkage group, corresponding to potato chromosome *11* including the locus involved in PCN resistance gene was recalculated with RECORD (van Os et al. 2005a), to assess putative ambiguities in marker order. With the information on marker order, the raw data were inspected for spurious data points (singletons). Singletons are easily recognised in graphical genotypes that are generated from the raw data in MS-Excel, where cell colour is formatted conditional to marker observation and linkage phase.

Data analysis

QTL analysis of the resistance data was performed with MapQTL 5.0 (van Ooijen and Maliepaard 1996). Data were transformed by taking the natural logarithm of the average cyst number (+1) per genotype, to obtain a uniform distribution of the variance. The heritability was estimated as follows: $h^2 = \sigma_g^2/\left(\sigma_g^2 + \sigma_e^2/n\right)$, where n is the number of replications, σ_e^2 is the expected mean square of the residuals and σ_g^2 is retrieved from the ANOVA table as follows: $\mathrm{E(MS)genotypes} = n\sigma_g^2 + \sigma_e^2$.

Two parametric methods: interval mapping and multiple-QTL mapping (MQM) were applied. The threshold for assigning a QTL was determined by a permutation test, as implemented in MapQTL 5.0.

Classification of cyst counts into resistant/susceptible phenotypes

For the qualitative mapping of the resistance locus, data of the first nematode resistance assay were used. All genotypes up to a maximum mean of 8 newly formed cysts were assigned as resistant and genotypes with at least 25 newly formed cysts were assigned as susceptible.

Results

The inheritance of PCN resistance

In an evaluation of tuber bearing *Solanum* species by The Centre for Genetic Resources (CGN), it was detected that *S. tarijense* accession BGRC 24717 contained resistance

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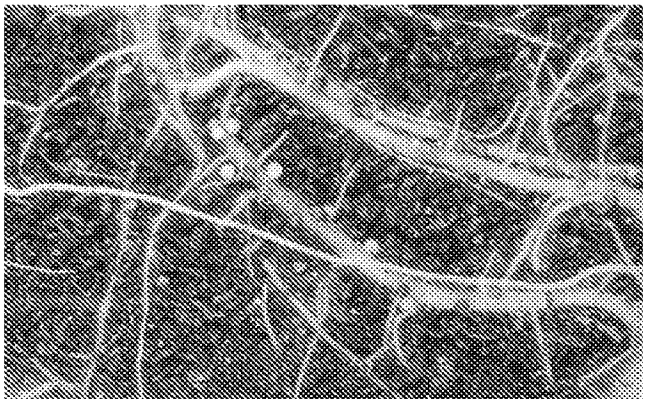


Fig. 2 Swollen females (appearing as *white cysts*) and *brown cysts* of *G. pallida* formed on roots of a susceptible potato plant

against *G. pallida* pathotype Pa3 (http://www.cgn.wur.nl). The inheritance of *G. pallida* resistance was analysed by testing a segregating BC1 population RHAM-061 of *S. tarijense* (BGRC 24717). Plant material inoculated with *G. pallida* population Rookmaker clearly showed a continuous distribution of the average number of cysts developed per genotype, ranging from 1 to 419. The resistant F1 hybrid parent RH90-011-4 developed on average 2 cysts while the susceptible *S. tuberosum* parent RH89-039-16 developed on average 132 cysts. An example of cysts on a susceptible plant is shown in Fig. 2. Fourteen genotypes of RHAM-061 with highly variable number of cysts in the different replications were excluded from further analysis. The variance in average number of cysts per genotype greatly differed between resistant and susceptible genotypes. A uniform distribution of the variance was obtained by taking the natural logarithm of the average cyst number per genotype. Analysis of the variance within and between genotypes showed that the genotypes differed significantly ($P < 0.001$). Broad sense heritability was estimated to be 0.82.

To test if the working spectrum of the *G. pallida* resistance also included *G. rostochiensis* resistance, the *S. tarijense* derived F1 parent RH90-011-4 was inoculated with *G. rostochiensis* in a container test. With all pathotypes, however, (Ro1, Ro2_3 and Ro5), large number of cysts were developed (between 20 and 60 cysts). The resistance originating from *S. tarijense* against *G. pallida* Rookmaker is therefore not also functional against *G. rostochiensis*.

Map construction

A linkage map was constructed using the segregating markers of 14 *Eco*RI/*Mse*I primer combinations. In total 518 segregating markers were identified. As much as 298 AFLP markers segregated from maternal genotype RH90-011-4, 155 markers from paternal genotype RH89-039-16. There were 65 bridge-markers which are heterozygous in both parents, which were used to connect maternal and paternal linkage groups as identified on the basis of the 1:1 segregating maternal or paternal marker loci. The map of maternal clone RH90-011-4 comprised 12 linkage groups with a total map length of 830.3 cM. The markers of paternal clone RH89-039-16 (RH) were assigned into 12 linkage groups with a total length of 561.1 cM. As much as 19 AFLP markers remained unassigned at LOD = 3. Chromosome numbers of RH were identified by aligning the RH linkage groups from this mapping population with the RH linkage groups of reference maps in our lab (Rouppe van der Voort et al. 1997a, 1998b; van Os et al. 2006). The 65 bridge-markers were sufficiently equally distributed over the 24 linkage groups and allowed us to pair all 12 maternal linkage groups to their homologous paternal (RH) linkage groups, hereby also identifying the chromosome numbers and orientations of the maternal linkage groups.

QTL mapping

The distribution of the ln-normalised cyst counts of the disease test of RHAM-061 was clearly bimodal which could be indicative of a large-effect QTL involved in quantitative resistance. QTL analysis was applied on all maternal and paternal linkage groups to identify all possible resistance factors. With the interval mapping method of MapQTL, one large-effect QTL was detected on maternal chromosome *11* (LOD = 57), which explained 81.3% of the phenotypic variance. No other marker—trait associations exceeded the LOD thresholds of the individual linkage groups as determined by the permutation test. To enhance the power to detect minor-effect QTLs, the MQM mapping method was applied with the QTL on chromosome *11* as a cofactor. This resulted in the identification of a QTL (LOD = 4.4) on the long arm of chromosome *9*. The percentage of explained phenotypic variance of this QTL was only 5.3%. Because of the small effect we assume that the resistance is essentially monogenically inherited and located on chromosome *11*.

Qualitative mapping of resistance

In view of the single locus involved in PCN resistance, as identified with QTL analysis, we proceeded with a qualitative genetic approach. The disease test phenotypes were used for a tentative classification of the offspring, where 76 genotypes containing on average of less than 8 cysts (ln 8 + 1 = 2.2) were assigned as resistant and 64 genotypes with an average of more than 25 (ln 25 + 1 = 3.3) were assigned as susceptible. The remaining 34 genotypes

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of intermediate phenotype were excluded from further analysis (Fig. 3a). The transmission of the resistance from the wild species to the BC1 mapping population, and the observed segregation ratio from the resistant parent, which is consistent with a 1:1 distribution ($\chi^2 = 0.067, P = 0.80$) allowed us to conclude that the effects can be explained by a single dominant gene.

The qualitatively segregating resistance data were added to the maternal marker dataset. According to JoinMap analysis, the resistance locus showed linkage with 10 markers on chromosome *11* in the same region, where the resistance QTL was previously mapped. The marker order of chromosome *11* including the resistance locus was verified with RECORD. The resistance locus, which we propose to name $GpaXI^{l}_{tar}$, is localised on a distal position on the long arm of chromosome *11* between AFLP markers eACTmCAA_174 and eAGAmCTG_222.

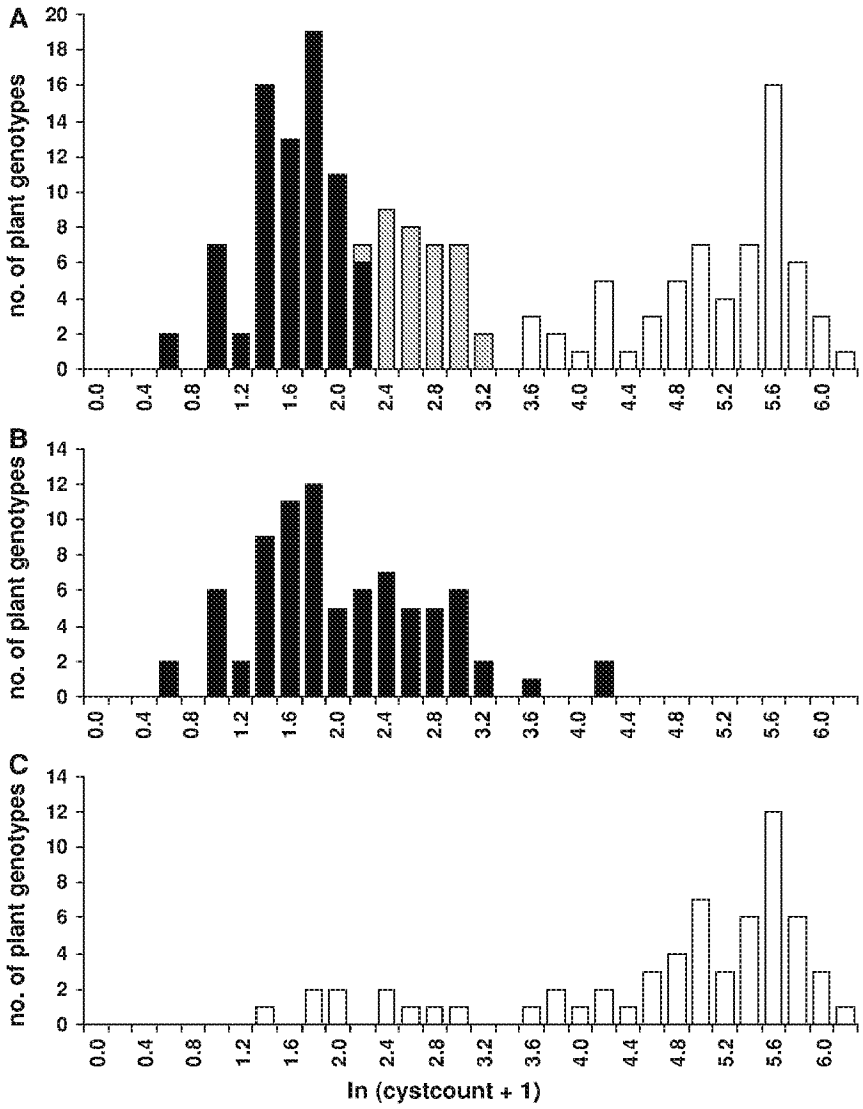


Fig. 3 Histograms depicting the distributions of the average natural logarithms of cyst counts (+1) of the individuals of mapping population RHAM-061 after inoculation with *G. pallida* population Rookmaker (Pa3). **a** Histogram of all the individuals of mapping population RHAM-061. *Black* genotypes tentatively assigned as resistant; on average of less than 8 cysts. *Grey* genotypes not assigned; on average between 9 and 24 cysts. *White* genotypes tentatively assigned as susceptible; on average more than 25 cysts. This classification of genotypes into the classes resistant/susceptible allowed mapping of the locus between flanking AFLP markers. **b** Histogram depicting the distribution of RHAM-061 genotypes where both flanking AFLP markers eACTmCAA_174 and eAGAmCTG_222 suggest the presence of resistance. Clones with recombination events between the markers were excluded from the figure. **c** Histogram depicting the distribution of RHAM-061 genotypes where both flanking AFLP markers eACTmCAA_174 and eAGAmCTG_222 suggest the absence of resistance. Clones with recombination events between the markers were excluded from the figure

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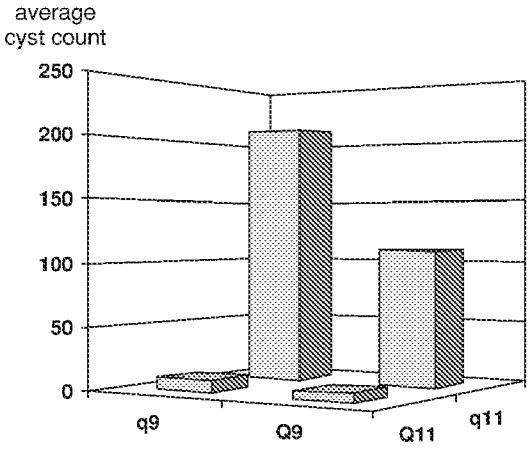
In order to validate the classification and the resulting map location, the flanking AFLP markers can be used to predict in retrospect the absence or presence of the resistance gene. When classifying on the basis of the flanking AFLP markers eACTmCAA_174 and eAGAmCTG_222 linked with resistance, the resistant group had on average 9.0 cysts, while the susceptible group had on average 148.9 cysts. The expected resistance, based on the prediction, was compared with the observed phenotypes (Fig. 3b, c). Judging on the basis of the flanking AFLP markers, 8 genotypes out of a total of 140 genotypes showed cyst counts that were incongruent with the expected presence/absence of the resistance gene, and were putatively misclassified. Four of these misclassifications concerned genotypes with no recombination event between the markers on the long arm of maternal chromosome *11*. The resistance phenotype of these descendants should be considered as false positives or negatives, without consequences for the position of the *R*-gene. The other four descendants can be best explained by assuming misclassification of the absence/presence of the flanking AFLP marker eAGAmCTG_222.

Interaction between $GpaXI^{l}_{tar}$ and the QTL on chromosome *9*

After identifying the two resistance QTL on chromosomes *9* and *11*, the magnitude of the individual effects and the possible interaction between the two QTL was analysed using ANOVA. The flanking AFLP markers eACTmCAA_174 and eAGAmCTG_222 linked with the $GpaXI^{l}_{tar}$ locus were used as explaining variable, indicative for the *R*-gene on chromosome *11*. AFLP marker eACAmCCA_26 showed the closest linkage with the QTL on chromosome *9* and was used as explaining variable indicative for the minor-effect QTL. Analysis of variance demonstrated (as expected) significant main effects for both QTL, but also a significant interaction effect between the QTL of chromosomes *9* and *11*. This interaction effect is best explored by illustrating the 2×2 interaction of both QTL alleles in Fig. 4, where the cyst counts of these four groups are shown. The group with the susceptible alleles for both $GpaXI^{l}_{tar}$ and the QTL on chromosome *9* showed an average cyst count of 210 cysts ($n = 29$). The group with the resistant allele of the QTL on chromosome *9* but the susceptible allele of $GpaXI^{l}_{tar}$ had an average cyst count of 109 ($n = 32$). This represents a significant decrease of 48% in the development of cyst numbers (2-sided *t*-test on ln (cyst count + 1), $df = 43$, $P = 0.00012$). In the presence of $GpaXI^{l}_{tar}$ the minor QTL had no statistically significant effect (2-sided *t*-test on ln (cyst count + 1), $df = 75$, $P = 0.14$). If the combination of the two QTL would have resulted in an additive effect, the expected average cyst count should be 5. Therefore, it is concluded that $GpaXI^{l}_{tar}$ displays a dominant epistatic interaction over the minor QTL.

Anchoring of $GpaXI^{l}_{tar}$ to a well-known *R*-gene cluster

The long arm of potato chromosome *11* is known to harbour several resistance genes. In order to determine the position of $GpaXI^{l}_{tar}$ relative to these genes, an attempt was



QTL composition of groups	average cyst count	standard error of the mean
qq qq	210.1	19.1
qq Q9q	109.3	17.0
Q11q qq	9.9	1.9
Q11q Q9q	6.7	0.9

Fig. 4 The reproduction of *G. pallida* Rookmaker (Pa3) in relation to the genetic composition of potato genotypes of the RHAM-061 mapping population. The height of the bars represent the average number of *G. pallida* Rookmaker (Pa3) cysts in the four QTL offspring classes. The presence or absence of $GpaXI^{l}_{tar}$ is determined by flanking AFLP markers eACTmCAA_174 and eAGAmCTG_222. Presence/absence of the QTL on chromosome *9* was identified by AFLP marker eACAmCCA_26. Q9 = resistant allele of the QTL on chromosome *9*. q9 = susceptible allele of the QTL on chromosome *9*. Q11 = resistant allele of $GpaXI^{l}_{tar}$. q11 = susceptible allele of $GpaXI^{l}_{tar}$

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made to generate a better resolution and to add reference markers, which are known to reside close to the *R*-gene cluster.

To screen for recombinants, DNA of 324 newly sown genotypes from RHAM-061 was genotyped with the flanking AFLP markers eACTmCAA_174 and eAGAmCTG_222. Recombinant genotypes were identified and subjected to a disease test. For 32 genotypes we obtained phenotypic data allowing identification of the position of $GpaXI^{I}_{tar}$ relative to markers GP163 and FEN427. In the complete population of 324, no recombination events were detected between markers GP163 and FEN427. Both markers mapped 1.3 cM proximal of $GpaXI^{I}_{tar}$. The position of GP163 and FEN427 relative to $GpaXI^{I}_{tar}$, confirms that $GpaXI^{I}_{tar}$ maps to a well-known *R*-gene cluster on the long arm of chromosome *11*. The resulting genetic map is presented in Fig. 5, also showing reference maps from literature used for anchoring purposes. As much as 17 of the 324 offspring clones (=5%) were excluded from map construction. In these plants we observed two recombination events at close distance, suggesting data point(s) that are in conflict with both their flanking markers (singletons). In view of chiasma interference these events are more likely to represent data error than true recombination events and can be removed safely without influencing marker order (van Os et al. 2005b). Even if these were real recombination events, the map distances would be hardly affected in view of the total population size of 324 offsprings.

Discussion

The inheritance of resistance to the white potato cyst nematode *G. pallida* has initially been regarded as complex (Dale and Phillips 1982; Ross 1986). With the aid of molecular markers it has been demonstrated that the allegedly complex resistance to *G. pallida* is simpler,

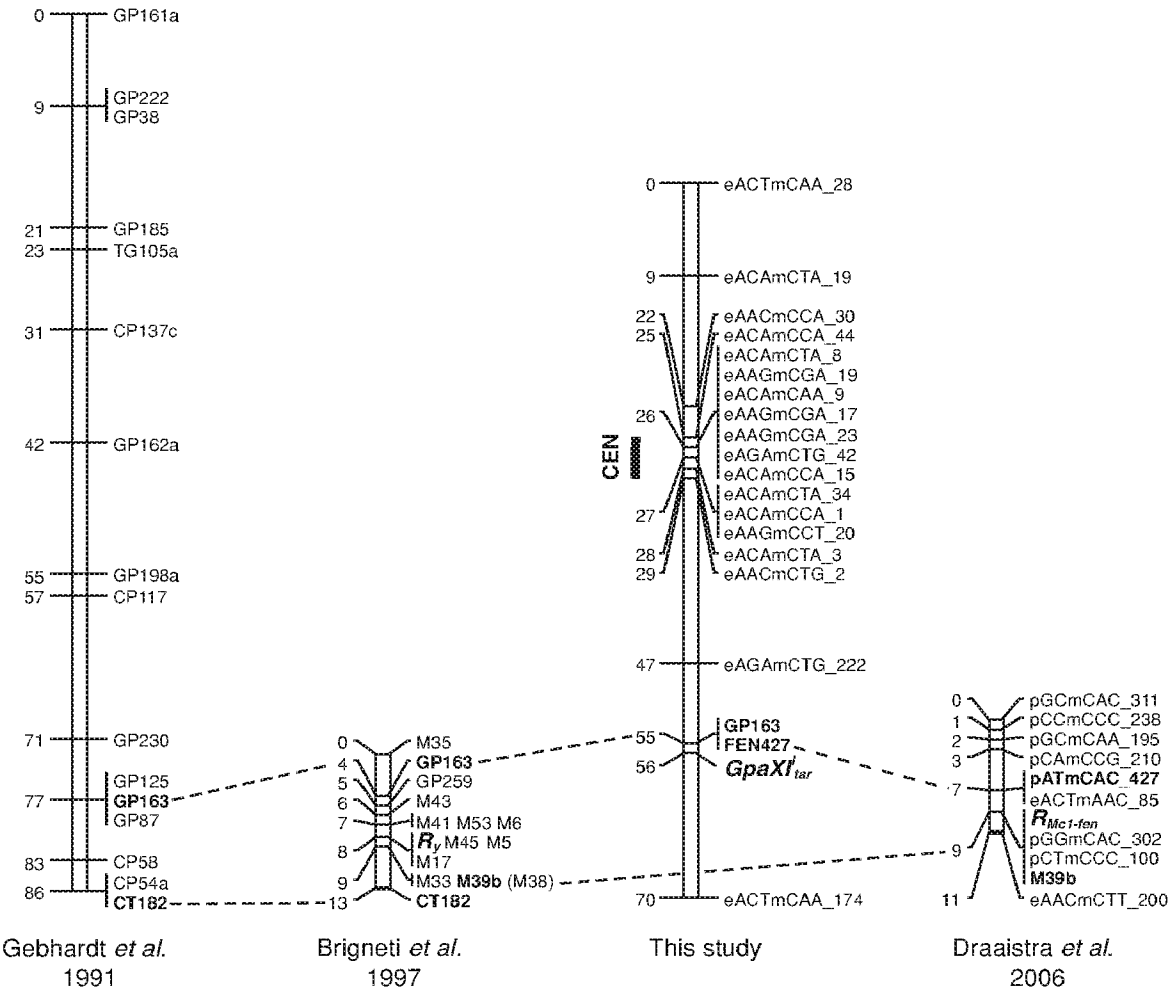


Fig. 5 The genetic map of maternal chromosome *11* of mapping population RHAM-061 showing the $GpaXI^{I}_{tar}$ locus in connection to chromosome *11* reference maps from literature used for anchoring purposes. Chromosome orientation is according to Dong et al. (2000)

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because in many cases large-effect QTL have been identified. For example, the QTL *Grp1*, *Gpa*, *Gpa5* and $GpaV^{s}_{spl}$ (Caromel et al. 2005; Kreike et al. 1994; Rouppe van der Voort et al. 1998a, 2000) invariably accounted for more than 45% of the phenotypic variance. In this study two loci have been mapped: a large-effect QTL derived from the wild *Solanum* species *S. tarijense* BGRC 24717, as well as a minor-effect QTL on potato chromosome *9*.

Identification of the large-effect resistance locus $GpaXI^{l}_{tar}$

A large-effect QTL has been identified on potato chromosome *11* explaining 81.3% of the phenotypic variance of the disease test. In view of the magnitude of the explained variance, the QTL was Mendelised. This allowed us to estimate the genetic distances between the locus and the flanking molecular markers and to place the locus name on the genetic map. Usually, a broad QTL interval does not offer precise ordering information of markers relative to the QTL, but in our case the order of the *R*-gene and the markers is regarded as a stable order, well supported by recombination events.

In the studied mapping population RHAM-016, the clones with the resistance allele $GpaXI^{l}_{tar}$ (as predicted by the flanking markers) developed on average 9.9 cysts, whereas clones without any resistance allele developed on average of 210 cysts. This constitutes a relative decrease of 95% in cyst count. The level of resistance obtained with $GpaXI^{l}_{tar}$ is therefore partial and not absolute. This non-absolute level of resistance seems to be common for most of the identified major loci involved in *G. pallida*. As discussed by Rouppe van der Voort et al. (1998a, 2000), the number of newly formed cysts in spite of the presence of a major *R*-gene, is likely due to heterogeneity in the *G. pallida* population Rookmaker (Pa3) at the respective (a) virulence gene. A single *R*-gene which operates on a gene-for-gene basis will confer partial resistance against the population as a whole, whereas on the level of the individual, the *R*-gene will confer absolute resistance against the matching avirulent genotype. Alternatively, it is also possible that the resistance mechanism of the *R*-gene in itself is not absolute. This has been demonstrated for the *H1* gene, where always a small number of cysts are formed on *H1* resistant plants after inoculation with a homozygous avirulent line of *G. rostochiensis* (Janssen et al. 1990). These cysts that are formed by a non-absolute resistance mechanism of a major *R*-gene are known as "escapers" and do not imply any kind of evolution of the nematode population towards increasing virulence. Whether the newly formed cysts on $GpaXI^{l}_{tar}$ resistant plants have been caused by the heterogeneity of the test population or by the strength of the *R*-gene itself can only be determined by testing the *R*-gene with a homozygous avirulent nematode population.

The resistant *S. tarijense* hybrid, which was used as parent of the mapping population showed an average cyst count of 2. Even when both resistance factors were present, the major locus $GpaXI^{l}_{tar}$ and the minor QTL on chromosome *9*, offspring clones did not achieve the same resistance level as their resistant parent. Apparently, there is an interaction between the *R*-gene(s) and the genetic background in which an *R*-gene is introgressed.

Identification of a small-effect resistance locus on chromosome *9*

Besides the major QTL on chromosome *11*, a minor QTL was detected on chromosome *9*. At this moment we can only speculate on the function of this QTL.

The minor QTL could represent a classic NBS-LLR gene, but also a factor in non-necrogenic resistance, for instance in the formation of hatching substances. Finally, the QTL could represent a locus involved in the size of the root system. As demonstrated by Kreike et al. (1994) a root size QTL will initially appear as a QTL for resistance. The dominant epistatic interaction of $GpaXI^{l}_{tar}$ over the minor QTL does not exclude one of the three possible explanations.

Both loci map to well-known *R*-gene clusters

Two CAPS markers GP163 and FEN427 co-localised and mapped 1.3 cM proximal of $GpaXI^{l}_{tar}$. Marker GP163 was previously positioned at a distance of 3.7 cM proximal to potato virus Y resistance gene Ry_{sto} on chromosome *11* (Brigneti et al. 1997). AFLP marker pATmCAC_427, on which CAPS marker FEN427 was based, was previously used to map nematode resistance $R_{Mc1\text{–}fen}$ on chromosome *11* and mapped 2 cM proximal of $R_{Mc1\text{–}fen}$ (Draaistra 2006). Therefore, we postulate that $GpaXI^{l}_{tar}$ is located in one and the same *R*-gene cluster, which is already comprising three nematode resistance genes $R_{Mc1\text{–}blb}$, $R_{Mc1\text{–}fen}$, $R_{Mc1\text{–}hou}$, (Draaistra 2006), as well as several more resistance genes against viruses and fungi (Gebhardt and Valkonen 2001). From this *R*-gene cluster only *N* (Whitham et al. 1994) has been cloned, suggesting that $GpaXI^{l}_{tar}$ could represent a TIR-NBS-LRR gene with homology to *N*. Future research may demonstrate the relation between $GpaXI^{l}_{tar}$ and the other nematode resistance genes in this cluster. Each of these nematode resistance genes could be involved in recognition of distinct effector molecules, but might also represent a casus similar to the *Mi* gene, recognising the nematode *Meloidogyne incognita* and the aphid *Macrosiphum euphorbiae* (Rossi et al. 1998; Vos et al. 1998).

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Likewise, the minor QTL described in this study maps in close proximity to a known *R*-gene cluster. This cluster on chromosome *9* comprises the *Sw-5* resistance gene (Brommonschenkel et al. 2000), a homologue of *Mi*. Interestingly, this cluster is already implicated in *G. pallida* resistance, as the small-effect *Gpa6* QTL was mapped here (Rouppe van der Voort et al. 2000). The coincidence of finding the minor QTL at a position indistinguishable from the *Gpa6* locus prompted us to verify the pedigree of our mapping population for the putative introgression segments of *S. vernei*. In Fig. 1, a crossing parent MON-46 is shown, a dihaploid of cv. Mondial. This parent is susceptible for *G. pallida*, but it has *S. vernei* in its pedigree. Therefore, it remains inconclusive if the minor QTL originates from *S. tarijense* or represents a gene identical by descent to *Gpa6* from *S. vernei*. Thus, we have refrained from giving the minor-effect QTL discovered on chromosome *9* a new name.

Mapping strategy

The identification of the genetic locus involved in a phenotypic trait can be achieved with various mapping strategies, but a minimal requirement is the ability to align the newly mapped locus with previous linkage studies. This can be achieved with single copy locus specific markers, but in this study the high multiplex efficiency of AFLP fingerprinting was used. With 14 *Eco*RI/*Mse*I AFLP primer combinations, 518 marker loci were obtained, which was sufficient to cover all 12 maternal and paternal linkage groups, as well as markers bridging the homologous parental linkage groups. Our strategy specifically exploited the principle that AFLP fragments of the same mobility on gel represent the same genetic map position, because of DNA sequence homology of the underlying DNA fragment captured in the AFLP fingerprint (Rouppe van der Voort et al. 1997b). This approach has been successful not only in our own hands with proper control samples in the same AFLP gel image (Rouppe van der Voort et al. 1997a), but also across labs (Bradshaw et al. 2006). The approach followed in this study, not only used the well-known reference genotype RH89-039-16 as a reference sample in AFLP fingerprinting, but it also served as susceptible crossing partner. The resistance locus, however, segregated from a wild species donor. The validity of locus-specificity of AFLP markers rapidly disappears with increasing taxonomic distance; as increasing taxonomic distance results in increasing AFLP fingerprint dissimilarity, where only insignificant coincidental co-migration remains. The obvious success to align the introgression of *S. tarijense* with the potato reference maps is due to the BC1 structure of the mapping population. For mapping purposes one could also use the F1, as both parents *S. tuberosum* and *S. tarijense* are non-inbred. A mapping population of F1 RH90-011 siblings would result in a maternal and paternal map, but the paternal *S. tarijense* linkage groups would be comprised of markers which might not bear resemblance to known markers in our database. The next generation (BC1) results in linkage groups, which are comprised of known marker alleles linked in repulsion to the resistance gene. Hence, mapping of the *R-gene* locus can be achieved irrespective of genetic dissimilarity between the wild species and potato.

Nomenclature of nematode resistance genes

At this moment the nomenclature of genes involved in nematode resistance is without consensus (Gebhardt and Valkonen 2001). On the one hand there is a series of major and minor-effect QTL against *G. pallida* with names ranging from *Gpa*, *Gpa2* until *Gpa6*, which are numbered successively following its order of identification. Likewise a series of *R*-genes and QTL against *G. rostochiensis* are known: *H1*, *Gro1*, *Gro1.2*, *Gro1.3*, *Gro1.4* and *GroV1* (Gebhardt and Valkonen 2001). Although such names nicely reflect history of science, it is hardly informative on the wild species origin, the resistance spectrum to various pathotypes, or the genetic location of the gene.

At this moment we wish to follow the syntax of Caromel et al. (2005) [pathogen species, potato linkage group (roman), long/short arm (superscript), source species (subscript)], and propose to name this *G. pallida* resistance $GpaXI^{l}_{tar}$. At the 'Symposium on the Molecular Biology of the Potato' held in 1998 at Bogensee, Germany, two large-effect QTLs were mentioned. An abstract by Wolters et al. (1998), describes *Gpa3* on chromosome *11* derived from *S. tarijense* and *Gpa4* on potato chromosome *5* derived from *S. sparsipilum*. We propose that *Gpa3* and *Gpa4* will no longer be used in scientific literature. *Gpa3* was mapped in an F1 segregating population different from our population. But since the same BGRC accession has been used as a source, it is very plausible that $GpaXI^{l}_{tar}$ is identical to *Gpa3*. Whereas *Gpa3* and *Gpa4* have not yet entered the peer-reviewed literature, *Gpa4* has already caused some confusion as it appeared in the review by Gebhardt and Valkonen (2001), where *Gpa4* was used for the unnamed QTL on potato chromosome *4* segregating from SCRI clone 12601ab1 (Bradshaw et al. 1998). The most plausible name for this QTL is the *H3* gene from *Solanum tuberosum* spp. *andigena* CPC2802 (p. 115 http://www.scri.ac.uk/scri/file/fullannualreports/annual_report_2001.pdf).

Practical value of the *G. pallida* (Pa3) resistance gene $GpaXI^{l}_{tar}$ in potato breeding

The resistance level of $GpaXI^{l}_{tar}$ (a relative decrease of 95% in cyst count) is comparable with the level conferred

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by *Grp1* (Rouppe van der Voort et al. 1998a) and *Gpa5* (Rouppe van der Voort et al. 2000); both resistance genes that are presently used in commercially grown PCN resistant cultivars. We therefore state that $GpaXI^{t}_{tar}$ will be an equally valuable gene for the development of PCN resistant cultivars. A first demonstration of the value of the *H3* gene is cv. Vales Everest, which is currently the best PCN resistant cultivar on the UK National List.

In contrast to the limited durability of *R*-genes in many other plant–pathogen interactions, several aspects of the potato–PCN interaction raise the support of a relatively longer durability of the major resistance genes. The multiplication rate and the spread of PCN are limited, and the time between generations can range from 3 to 5 years depending on crop rotation schemes. Changes towards new virulent PCN types are therefore likely to be slow. Furthermore, the positive selection of virulent factors will be countered by the obligate sexual reproduction with genetically heterogeneous males.

Nowadays PCN resistant cultivars are available. However, because of the partial effect of the used *R*-genes a wide crop rotation is still required. As discussed before, the partial effect of the resistance genes can be explained by the genetic heterogeneity of the *G. pallida* field populations and by "escapers" that are caused by an ineffective resistance mechanism. The ideal situation for potato growers would be an absolute resistance in one cultivar. Possibly such an absolute resistance level could be achieved by combining or "pyramiding" of different *G. pallida* resistance genes. The development of breeding material with more than one allele at a given locus seems ineffective (Brodie and Plaisted 1992). Clones with a combination of major-effect QTL $GpaV^{s}_{spl}$ and minor-effect QTL $GpaXI^{s}_{spl}$ showed additively lower cyst counts than with the individual QTL (Caromel et al. 2005). The same was shown with a combination of major-effect QTL *Gpa5* with minor-effect QTL *Gpa6* (Rouppe van der Voort et al. 2000). Both studies showed lower cyst counts when combining a major and a minor QTL, but no absolute resistance. It would be interesting whether combining major QTL could result in absolute resistance. It seems that a combination of *R*-genes with different resistance spectra should at least make it possible to efficiently tackle the problem of heterogeneity of the *G. pallida* populations.

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ORIGINAL PAPER

The effect of pyramiding *Phytophthora infestans* resistance genes $R_{Pi\text{-}mcd1}$ and $R_{Pi\text{-}ber}$ in potato

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Abstract Despite efforts to control late blight in potatoes by introducing R_{pi}-genes from wild species into cultivated potato, there are still concerns regarding the durability and level of resistance. Pyramiding R_{pi}-genes can be a solution to increase both durability and level of resistance. In this study, two resistance genes, $R_{Pi\text{-}mcd1}$ and $R_{Pi\text{-}ber}$, introgressed from the wild tuber-bearing potato species *Solanum microdontum* and *S. berthaultii* were combined in a diploid *S. tuberosum* population. Individual genotypes from this population were classified after four groups, carrying no R_{pi}-gene, with only $R_{Pi\text{-}mcd1}$, with only $R_{Pi\text{-}ber}$, and a group with the pyramided $R_{Pi\text{-}mcd1}$ and $R_{Pi\text{-}ber}$ by means of tightly linked molecular markers. The levels of resistance between the groups were compared in a field experiment in 2007. The group with $R_{Pi\text{-}mcd1}$ showed a significant delay to reach 50% infection of the leaf area of 3 days. The group with $R_{Pi\text{-}ber}$ showed a delay of 3 weeks. The resistance level in the pyramid group suggested an additive effect of $R_{Pi\text{-}mcd1}$ with $R_{Pi\text{-}ber}$. This suggests that potato breeding can benefit from combining individual R_{pi}-genes, irrespective of the weak effect of $R_{Pi\text{-}mcd1}$ or the strong effect of $R_{Pi\text{-}ber}$.

Introduction

Late blight, caused by *Phytophthora infestans*, is a ruthless disease causing great losses in potato yield. One of the solutions has been the use of chemicals. The International Potato Centre (CIP) estimates that about 1 billion dollars per year are spent on fungicides to control late blight in the US, Europe and developing countries (http://gilb.cip.cgiar.org/what-is-late-blight/economic-impact/social-impact-and-economic-importance-of-late-blight/). The loss in yield is nevertheless still considerable, and in addition, the environment suffers from the use of fungicides. In the 1900s, the first successful breeding efforts were undertaken to raise resistance levels of potato varieties by hybridization with wild potatoes (Salaman 1910). Eleven R_{pi}-genes (*R1–R11*), introgressed from *S. demissum*, were the first ones to be used in potato cultivars. But unfortunately soon after the introduction of many potato cultivars with *S. demissum* *R*-genes in the 1950s and 1960s, the resistance proved not to be durable. For a period of time, the prevailing strategy to achieve a durable solution against *P. infestans* was thought to be the use of horizontal (race-non-specific) resistance instead of *R*-genes in potato breeding (Colon et al. 1995; Landeo et al. 1995; Tan et al. 2008; Turkensteen 1993). Potato genotypes showing a hypersensitive response to *Phytophthora* races were discarded, because the non-durable *R*-genes would only blur the view on the level of horizontal resistance. Breeding for late blight resistance would require "R_{pi}-gene free" germplasm (Turkensteen 1993). This strategy has not provided any improvement, mostly because of the inevitable correlation between resistance and late maturity (Simko 2002). Furthermore, it might be a valid assumption that residual effects of defeated R_{pi}-genes could be a factor in horizontal resistance (Stewart et al. 2003; Rauscher et al. 2008; Tan et al. 2008). These days the focus is back on R_{pi}-gene mediated resistance (Allefs et al. 2005). Since the discovery of the *S. demissum* *R*-genes, a wide range of wild *Solanum* species have been identified as potential sources for more R_{pi}-genes, e.g. species

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in the Dutch–German potato collection (Ross and Baerecke 1951; Van Soest et al. 1984). Eventually, the resistance genes $R_{Pi\text{-}ber}$ from *S. berthaultii* (Ewing et al. 2000), $R_{Pi\text{-}mcd1}$ from *S. microdontum* (Tan et al. 2008) and four R_{pi}-genes from *S. bulbocastanum*: *RB*/$R_{Pi\text{-}blb1}$, $R_{Pi\text{-}blb2}$, $R_{Pi\text{-}blb3}$ and $R_{Pi\text{-}abpt}$ (Park et al. 2005a, b; Song et al. 2003; van der Vossen et al. 2003, 2005) have been characterised. Furthermore R_{pi}-genes similar to $R_{pi\text{-}blb1}$ have also been found in *S. verrucosum* (Liu and Halterman 2006) and in *S. stoloniferum* and *S. papilla* (Vleeshouwers et al. 2008). Currently, these R_{pi}-genes are being deployed in breeding programs and one ($R_{Pi\text{-}blb2}$) is present in the commercial potato varieties "Toluca" and "Bionica" after more than 40 years of traditional crossing and breeding efforts (Hermsen 1966). However, not all of the R_{pi}-genes render absolute levels of resistance in the practical field situation. Worse is the expected durability of these newly identified R_{pi}-genes, as there is no reason to assume a longer durability of the new R_{pi}-genes than of *R1–R11*, especially since these new R_{pi}-genes have to oppose faster evolution rates of *P. infestans* with the introduction of the A2 mating type in the 1980s.

Pyramiding (major) *R*-genes can be one solution to improve on both durability and level of resistance (Nelson 1972). Pyramiding is the accumulation of (*R*)-genes into a single genotype or cultivar and can be achieved using major R_{pi}-genes, defeated R_{pi}-genes, different alleles of one gene, or the same alleles (allele-dosage). Although the potato cultivars such as Pentland Dell (1961) and Escort (1982) demonstrate that breeders were involved in pyramiding a long time ago, a surprisingly limited number of scientific reports have been published quantifying the effects of pyramided *R*-genes in different plant-pathosystems. Barloy et al. (2007) showed a higher level of resistance against cereal cyst nematodes in wheat when pyramiding resistance genes CreX and CreY. Several groups have reported on the pyramiding of bacterial blight resistance genes in rice and observed higher resistance levels and obtained additionally, a broader spectrum of resistance (Huang et al. 1997; Singh et al. 2001; Yoshimura et al. 1995; Zhang et al. 2006). When pyramiding multiple QTLs involved in resistance against barley stripe rust, Richardson et al. (2006) reported higher levels of resistance as achieved with individual QTLs. Whereas most studies show an additive effect of pyramiding two *R*-genes, the study of Sharma et al. (2004) did not. Marker-assisted pyramiding of the brown planthopper resistance genes *Bph1* and *Bph2* on rice chromosome *12* resulted in a resistance level of the pyramided line equivalent to that of the *Bph1*-single introgression line.

Slightly different are the examples of studies on the effect of allele-dosage. Toxopeus (1957) observed no additional *P. infestans* resistance in duplex R_3 or triplex R_3 genotypes compared to simplex R_3 genotypes. Likewise, no effect of allele dosage was observed in a study with H_1, an *R*-gene against *Globodera rostochiensis* in potato, where numbers of developed cysts were equal on genotypes containing simplex or multiplex copies of the H_1 gene (Brodie and Plaisted 1992).

In this paper, we describe a potato field trial in which the effect of pyramiding two *P. infestans R*-genes on late blight resistance is studied. The two *R*-genes $R_{Pi\text{-}ber}$ (chromosome *10*) and $R_{Pi\text{-}mcd1}$ (chromosome *4*) used for pyramiding have been described in literature. $R_{Pi\text{-}mcd1}$ is described in Tan et al. (2008). To study the effect of pyramiding, it is necessary that both used R_{pi}-genes do not provide absolute resistance. From our own experience with $R_{Pi\text{-}mcd1}$, an *R*-gene originating from *S. microdontum*, we knew that this *R*-gene gives a weak resistance to *P. infestans* in the field (Tan et al. 2008). Since both *R*-genes do not give absolute resistance, we deemed them suitable for studying putative quantitative differences in resistance when pyramiding these *R*-genes.

We developed a pyramiding population by crossing two diploid parents that were both heterozygous for one of the R_{pi}-genes. In theory, the full-sib offspring can be divided into four groups, carrying no R_{pi}-gene, with only $R_{Pi\text{-}mcd1}$, with only $R_{Pi\text{-}ber}$, and a group with the pyramided $R_{Pi\text{-}mcd1}$ and $R_{Pi\text{-}ber}$. The advantage of making one pyramiding population instead of comparing cultivars with different and combined R_{pi}-genes, is that the genetic background of the groups in one population is on average the same. The four groups cannot be differentiated by late blight assays, unless the different resistance spectra (if any) of the R_{pi}-genes can be used. However, because the genetic position of both *R*-genes $R_{Pi\text{-}ber}$ and $R_{Pi\text{-}mcd1}$ are known, we could make use of molecular markers flanking the *R*-genes. These molecular markers proved an accurate tool to genotype the pyramiding population and assign the genotypes into the four groups: (1) without R_{pi}-genes, (2) with $R_{Pi\text{-}mcd1}$ present, (3) with $R_{Pi\text{-}ber}$ present, and (4) containing both R_{pi}-genes $R_{Pi\text{-}mcd1} + R_{Pi\text{-}ber}$.

Materials and methods

Plant material and *Phytophthora* resistance assays

Two segregating diploid populations were developed. The first population RH97-739 (see Fig. 1) was developed to verify the map position of $R_{Pi\text{-}ber}$ and to develop flanking DNA markers. The second population RH03-424 (see Fig. 2) was developed to identify four groups of descendants without, with either or both R_{pi}-genes.

The first population, which was used to characterise the $R_{Pi\text{-}ber}$ gene used in this experiment was derived from *S. berthaultii* gene bank accession CGN 17823/PI265858. This gene might differ from the R_{pi}-gene from the *S. berthaultii*

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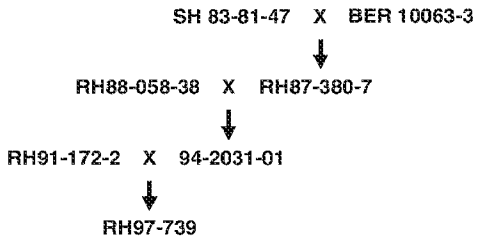


Fig. 1 Pedigree of RPi-ber mapping population RH97-739 used to develop flanking markers

gene bank accession PI473331 (Ewing et al. 2000 and Rauscher et al. 2006). To verify that the position of both R_{pi}-genes ($R_{Pi\text{-}berI}$ and $R_{Pi\text{-}ber}$) is identical, a disease assay was performed with the interspecific mapping population RH97-739 (Fig. 1). Eighty-eight genotypes were tested in a field experiment in 2005, as well as in a detached leaf assay, whereas the population size for marker analysis was 79 descendants. The field assay was performed in two replications, in a randomised complete block design. Inoculation took place with a spore suspension of a complex *P. infestans* isolate IPO-82001 (race 1,2,3,4,5,6,7,10,11) as described by Vleeshouwers et al. (1999). Infection was scored 18 and 30 days post inoculation (dpi). The genotypes were scored in a qualitative fashion at 30 dpi and were classified as either resistant when infection was $\leq$50% of the average leaf area or susceptible when $\geq$95% of the average leaf area was infected. The genotypes that fell in-between <50 and >95% were excluded from the qualitative genetic analysis. The detached leaf assay was performed with leaves collected from greenhouse plants and were tested in two replications as described by Vleeshouwers et al. (1999). The cultivar Bintje and parents RH91-172-2 and 94-2031-01 were included as control plants. Complex isolates IPO-82001 (race 1,2,3,4,5,6,7,10,11) and IPO-90128 (race 1,3,4,6,7,8,10,11) were used to determine leaf resistance. Inoculation was performed as described in Vleeshouwers et al. (1999) except that the concentration of the inoculum was adjusted to 5×10^5 zoospores/ml. In case of discrepancy between field and detached leaf assay results the field data were deemed most reliable.

The second population, also referred to as the pyramiding population RH03-424 ($n = 93$), descended from a cross between $R_{Pi\text{-}mcdI}$ donor RH90-038-21, a BC1 of *S. microdontum* accession BGRC 18302 (CGN21342), and $R_{Pi\text{-}ber}$ donor 94-2031-01, a BC1 of *S. berthaultii* accession BGRC 10063 (CGN 17823/PI265858). The pedigree of RH03-424 is presented in Fig. 2. The resistance gene $R_{Pi\text{-}mcdI}$ is located on the short arm of chromosome *4* and gives a delay of infection to *P. infestans* (Tan et al. 2008).

In 2007, a field assay was performed on 93 individuals of pyramiding population RH03-424. The location of the field was the "Hoge Born", in the area near Wageningen, NL on sandy soil. Experimental design was a randomised complete block design, in two replications. Per replication, 4 tubers per genotype were planted. Astarte, Bildtstar and Eersteling were used as standards. Tubers were planted on the 17th of April. Inoculation took place on the 26th of June with a spore suspension of a complex *P. infestans* isolate IPO-82001 (race 1,2,3,4,5,6,7,10,11) as described by Vleeshouwers et al. (1999). Foliage infection levels were scored 15 times during 6.5 weeks. The percentage of diseased leaf area was recorded on a scale comprising 16 classes, corresponding to 0, 1, 2, 5, 10, 20,..., 80, 90, 95, 99, 100 percent diseased leaf tissue. The four plants per genotype were scored as one experimental unit.

Maturity type was observed on a fungicide protected field experiment, on clay soil, in August of 2006 and 2007. Maturity type was scored approximately 130 days after planting on an ordinal scale ranging from 2 (green and vigorous; late to very late) to 6 (yellowing and plant is sagging) to 9 (dead; very early plant types). Four plants per genotype were treated as one experimental unit. Observations from 2006 and 2007 were averaged.

Maturity corrected resistance (MCR) was obtained essentially as described by Visker et al. (2003), where MCR is estimated from the initial resistance value using maturity as co-variate. MCR thus represents the difference between the observed resistance value and the resistance value as expected given its maturity. When resistance and maturity data from this population or from potato cultivars from the Dutch National List are plotted in a graph, the regression slope of their correlation is approximately -1. This means that one unit gain on the maturity scale is decreasing late blight resistance with one unit (Visker 2005). Therefore, MCR values (or residuals) can be obtained easily by subtracting the maturity value from the resistance value. To display MCR values in a graph along



Fig. 2 Pedigree of pyramiding population RH03-424 which combines none, either or both the R_{pi}-genes from *Solanum microdontum* and *S. berthaultii*

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with uncorrected resistance, the MCR values were increased with a constant value that equals the average maturity of the population. (MCR = foliage infection − maturity value + average maturity).

Genomic DNA extraction

Genomic DNA was isolated from young leaf material, harvested from young greenhouse grown plants, as described by van der Beek et al. (1992). Fresh tissue was ground at room temperature in STE-extraction buffer with steel balls using a Retsch machine (Retsch Inc., Haan, Germany). All volumes of the DNA extraction procedure were adapted to 2 ml deep 96-wells Costar plates (Corning Inc., Corning, NY, USA).

DNA marker assays

The verification and mapping of the $R_{Pi\text{-}ber}$ gene was performed with CAPS markers CT214 (Rauscher et al. 2006) and TG63 and SCAR marker Q133. TG63F2/R2 primers were newly developed on TG63 DNA sequence of a genotype of *S. berthaultii* accession BGRC 10063 (CGN 17823/PI265858) amplified with TG63F1/R primers (Rauscher et al. 2006). Marker Q133, was newly developed using DNA sequence information (Genbank AF404451) of a Resistance Gene Analogue which maps to chromosome *10* of tomato (Pan et al. 2001). Genotyping of the resistance locus $R_{Pi\text{-}mcd1}$ was performed with chromosome *4* specific CAPS marker TG339 (Tan et al. 2008). All primers were developed with Primer3 software (Rozen and Skaletsky 2000). Primers and PCR conditions are listed in Table 1. Subsequently, the flanking molecular markers were tested for usability on the parents of the pyramiding population because it might be possible that polymorphisms found in one population cannot be used in other populations. However, all markers were usable for genotyping RH03-424 with the restriction enzymes identified in the $R_{Pi\text{-}mcd1}$ or $R_{Pi\text{-}ber}$ mapping population. The molecular markers allowed the pyramiding population to be divided into four groups: (1) without R_{pi}-genes, (2) with $R_{Pi\text{-}mcd1}$ present, (3) with $R_{Pi\text{-}ber}$ present, and (4) containing both R_{pi}-genes $R_{Pi\text{-}mcd1} + R_{Pi\text{-}ber}$ pyramided.

Linkage map construction of $R_{Pi\text{-}ber}$

The marker order of chromosome *10* specific markers Q133, TG63, CT214 including $R_{Pi\text{-}ber}$ was determined by RECORD (van Os et al. 2005) and visually inspected by graphical genotyping the raw data in MS-Excel using the conditional formatting of cells conditional to marker genotype and linkage phase. Map distances were calculated based on the frequency of recombination between the markers, and in view of the short distances no adjustment for putative double cross-overs was made.

Statistical analysis

Analyses were performed with MS-Excel and comprised the chi-square test for goodness-of-fit and the analysis of group differences in resistance level in the pyramiding population using a 2-sided *t* test assuming unequal variances.

Results

Mapping of $R_{Pi\text{-}ber}$

The response of the genotypes of the $R_{Pi\text{-}ber}$ mapping population RH97-793 in the field assay could be scored in a discrete fashion, where 49 genotypes could be scored as resistant and 37 as susceptible. The vast majority of the susceptible clones reached infection within 18 dpi, whereas the resistant clones were largely without symptoms. Only at 30 dpi did the first few resistant genotypes start to display infection (see Fig. 3). Two genotypes could not be scored unambiguously. The detached leaf assay showed the same segregation pattern as compared with the field assay, except for four differences, probably misclassifications due to mildew infection in the detached leaf assay. Therefore,

Table 1 Overview of the markers, primers and PCR conditions used in this study

Marker	Primersequences 5′ → 3′	Annealing temperature and extension time	RE digestion
CT214	F: GAACGCGAAAGAGTGCTGATAG	Tm 60°C, 60 s	*Dde*I
	R2: CCCGCTGCCTATGGAGAG T		
TG63	F2: TCCAATTGCCAGACGAA	Tm 55°C, 75 s	*Bme*1390I
	R2: TAGAGAAGGCCCTTGTAAGTTT		
TG339	F:GCTGAACGCTATGAGGAGATG	Tm 56°C, 90 s	*Mnl*I
	R:TGAGGTTATCACGCAGAAGTTG		
Q133	F: ATCATCTCCTCAAAGAATCAAG	Tm 50°C, 30 s	
	R2: ATCTCCCCATTGACAACCAA		

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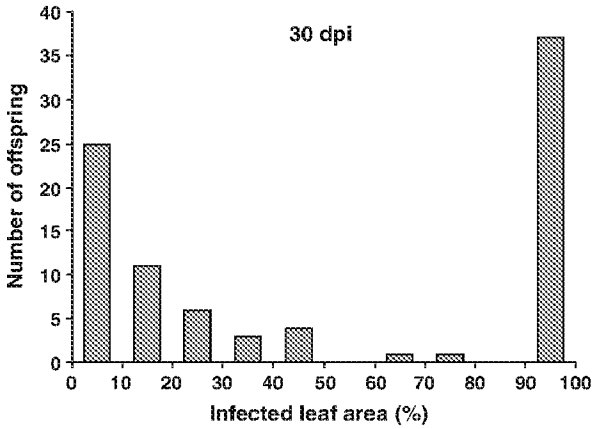


Fig. 3 Distribution of the percentage of infected leaf area at 30 days past infection of the offspring genotypes of the mapping population segregating for $R_{Pi\text{-}ber}$. From 17 until 30 dpi the U-shaped distribution allows unambiguous classification of resistant and susceptible segregants

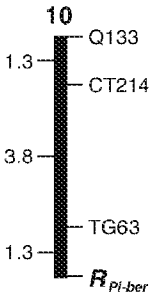


Fig. 4 Genetic map of part of chromosome *10* of paternal resistant parent 94-2031-01 including the location of $R_{Pi\text{-}ber}$

subsequent analyses have been based on results of the field assay. Chromosome *10* specific marker loci Q133, CT214 and TG63 are closely linked with the locus for resistance, at distances of 6.4, 5.1 and 1.3 cM, respectively, north of $R_{Pi\text{-}ber}$. The order and distances of the markers towards $R_{Pi\text{-}ber}$ was determined by five recombinants, and one singleton marker observation.

One of the five recombinants suggested the position of TG63 to be located north of $R_{Pi\text{-}ber}$. While taking this marker order, another descendant, with an ambiguous resistance phenotype then resulted in a singleton. Only the alternative marker order with TG63 south of $R_{Pi\text{-}ber}$ would remove this friction, but would cause map friction in the recombinant with an unambiguous late blight phenotype. Therefore, the marker order as shown in Fig. 4 is the most plausible order as judged from the marker data.

Analysis of the pyramiding population RH03-424 with markers flanking $R_{Pi\text{-}mcd1}$ and $R_{Pi\text{-}ber}$

To identify which of either resistance genes are present in each offspring of the pyramiding population ($n = 93$), molecular markers flanking the R_{pi}-genes were used. The pyramiding population RH03-424 could be differentiated into four groups: (1) without R_{pi}-genes, (2) with $R_{Pi\text{-}mcd1}$ present, (3) with $R_{Pi\text{-}ber}$ present, and (4) containing both R_{pi}-genes $R_{Pi\text{-}mcd1} + R_{Pi\text{-}ber}$ pyramided (Table 2). The observed segregation pattern into the four groups deviated significantly from the expected 1:1:1:1 ratio ($\chi^2 = 16.7$, $P < 0.001$). This deviation is largely due to an overrepresentation of resistant genotypes having the $R_{Pi\text{-}ber}$ allele (63:30), whereas the $R_{Pi\text{-}mcd1}$ locus hardly deviates from a 1:1 segregating ratio 56:37 ($\chi^2 = 3.88$; $P = 0.05$).

Table 2 Observed segregation of the descendants of the pyramiding population RH03-424 into four groups; without R_{pi}-genes, with $R_{Pi\text{-}mcd1}$ present, with $R_{Pi\text{-}ber}$ present, and containing both R_{pi}-genes $R_{Pi\text{-}mcd1} + R_{Pi\text{-}ber}$ according to the flanking molecular markers on chromosome *4* and *10*

	$+R_{Pi\text{-}ber}$	$-R_{Pi\text{-}ber}$
$+R_{Pi\text{-}mcd1}$	37	19
$-R_{Pi\text{-}mcd1}$	26	11

Evaluation of the resistance level in the offspring of the pyramiding population

The results described below will first address the effect of the individual genes (a) $R_{Pi\text{-}mcd1}$ and (b) $R_{Pi\text{-}ber}$, respectively, (c) their interaction and (d) the effects after correction for maturity type.

R_{pi}-gene free group versus $R_{Pi\text{-}mcd}$ group

One week after inoculation, the R_{pi}-gene free group had an average foliage infection of 66% (Fig. 5a). The group of descendants with the $R_{Pi\text{-}mcd1}$ allele showed infection as well, but to a lesser degree with 46% infection at this time point. The difference between the groups with and without the $R_{Pi\text{-}mcd1}$ allele was significant from 7 until 14 dpi (2-sided *t* test, $df = 24$–27, $P < 0.05$), with the largest effect at 9 dpi ($P = 0.0027$). The difference was no longer significant at 17 dpi and later time points, when both groups with or without $R_{Pi\text{-}mcd1}$ approached 100% infection.

Resistance level of $R_{Pi\text{-}ber}$

The difference in infection level between the groups with or without the $R_{Pi\text{-}ber}$ allele is much larger compared to the effect caused by the $R_{Pi\text{-}mcd1}$ allele (Fig. 5a). When the foliage of plants from the R_{pi}-gene free group is more than 90% infected, the $R_{Pi\text{-}ber}$ bearing groups remain almost free from *Phytophthora* symptoms, with an average degree of infection below 5%. This absence of *Phytophthora* infection as the effect of the $R_{Pi\text{-}ber}$ allele, remains until 24 dpi, a full 3 weeks longer than the R_{pi}-gene free group, as well as the $R_{Pi\text{-}mcd1}$ group. $R_{Pi\text{-}ber}$ does not, however, provide absolute

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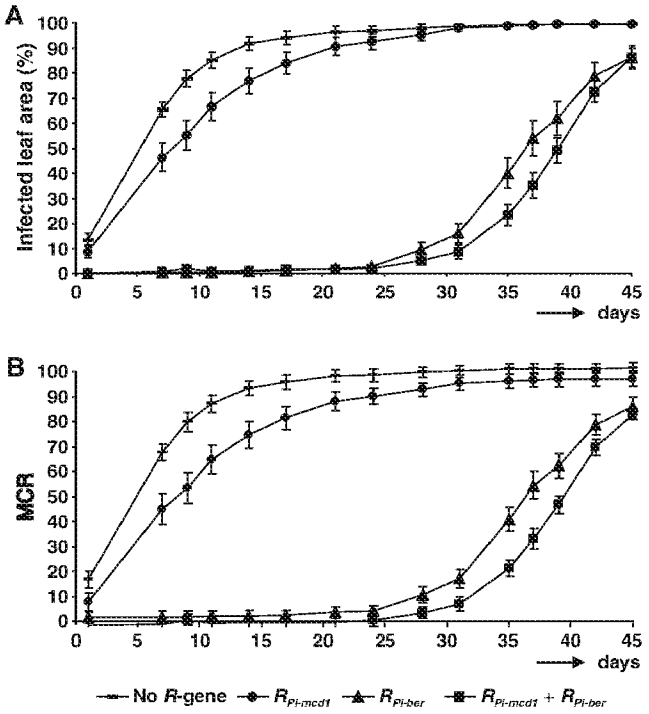


Fig. 5 Late blight disease progress curve showing the proportion of leaf area infected across 45 days post infection (dpi) of the four groups of pyramiding population RH03-424; without R_{pi}-genes, with $R_{Pi\text{-}mcd1}$ present, with $R_{Pi\text{-}ber}$ present, and containing both R_{pi}-genes $R_{Pi\text{-}mcd1}$ + $R_{Pi\text{-}ber}$. *Error bars* indicate the standard error of the mean. **a** Uncorrected disease progress using values as observed in the field. **b** Disease progress values using maturity corrected resistance (MCR) values

resistance. At 24 dpi, these plants also start to show late blight infection. In this experiment, the genotypes with $R_{Pi\text{-}ber}$ passed the level of 50% infection on average at 36 dpi, 6 times later than the R_{pi}-gene free genotypes and 4.5 times later than genotypes with $R_{Pi\text{-}mcd1}$.

Effect of pyramiding $R_{Pi\text{-}mcd1}$ + $R_{Pi\text{-}ber}$

From the first observation date, the presence of the $R_{Pi\text{-}ber}$ allele had a more noticeable effect as compared to the presence of the $R_{Pi\text{-}mcd1}$ allele. The effect of pyramiding of the two resistance genes can only be observed when the plants having the $R_{Pi\text{-}ber}$ allele (with or without $R_{Pi\text{-}mcd1}$) also showed infection. A significant contribution (2-sided *t* test, $df = 45–48$, $P < 0.05$) of the $R_{Pi\text{-}mcd1}$ allele in plants with $R_{Pi\text{-}ber}$ was observed from 35 dpi and remains significant till 37 dpi, with the largest effect observed at day 35 ($p = 0.026$) (Fig. 5a). Considering that the genetic background of the $R_{Pi\text{-}ber}$ group and the pyramided groups are on average the same, we conclude that the difference in resistance level was due to the additive effect of the $R_{Pi\text{-}mcd1}$ allele. The magnitude of this effect is comparable with the difference observed between the R_{pi}-gene free group and the $R_{Pi\text{-}mcd1}$ group: 50% infection was achieved 3 days later in the pyramided group than in the group containing only the $R_{Pi\text{-}ber}$ allele.

The effect of plant maturity on late blight infection

Within each group, there is considerable variation in the level of infection per genotype, where some genotypes containing the $R_{Pi\text{-}ber}$ allele may have a level of infection which is lower than some genotypes in the pyramided group. The infection of both the $R_{Pi\text{-}ber}$ groups begins at 24 dpi, which is 3 months after planting, long enough for first early cultivars to end their life cycle. Maturity types in the pyramiding population varied between 4 and 9 with a mean of 6.9 on a total scale of 1 to 9. Differences in maturity type could potentially influence the level of late blight resistance. The strong correlation between *P. infestans* foliage resistance and late plant maturity resulted in the use of maturity corrected resistance (MCR) (Bormann et al. 2004; Visker et al. 2004). Therefore, we re-analysed our late blight resistance observations using a maturity corrected dataset, where late blight resistance is compensated for the effect of maturity. MCR was calculated in this study by subtracting maturity values from leaf area infection values, but adding up the average value for maturity of the population, leaving the residuals. This is a simple and valid approach, because analysis of variance components showed that the variance contributed by maturity is of the same magnitude as the variance contributed by the R_{pi}-gene composition.

In Fig. 6, the effect is illustrated of this correction for maturity on late blight infection at 35 dpi. When comparing the maturity uncorrected (Fig. 6a) versus the maturity corrected (Fig. 6b) analysis, it becomes apparent that the correction for maturity slightly improves the difference in resistance levels between the $R_{Pi\text{-}ber}$ group and the $R_{Pi\text{-}ber}$ + $R_{Pi\text{-}mcd1}$ group. In addition, the correction for maturity

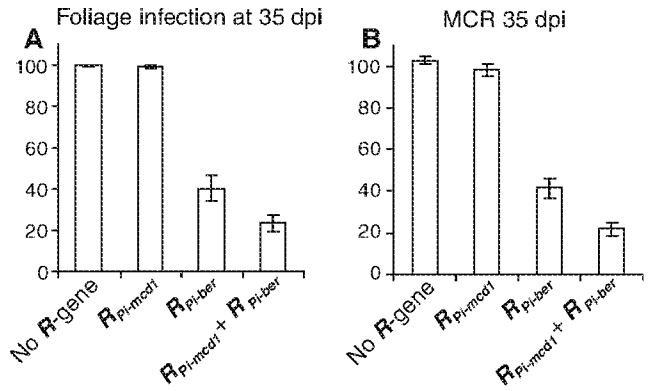


Fig. 6 Foliage infection (in %) of offspring genotypes classified according to R_{pi}-genes (**a**) Group averages of foliage infection at 35 dpi. **b** Group averages of maturity corrected resistance (MCR) at 35 dpi. *Error bars* indicate the standard error of the mean

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reduced the standard error of the group mean. Removing the heterogeneity in maturity between plants within the four groups thus allowed a more accurate assessment of late blight resistance differences due to R_{pi}-gene composition. The correction for maturity also confirmed that quantitative variation in the observed level of infection was not only caused by R_{pi}-genes but in part caused by maturity. Lastly, the maturity corrected analysis showed that the effect of pyramiding remains statistically significant for a much longer period, from 31 until 39 dpi (9 days) instead of 3 days (2-sided t test, $df = 47–50$, $P < 0.05$), with the largest effect at 35 dpi ($P = 0.0014$). In Fig. 5b, the progress in time of the maturity corrected foliage infection for the four groups is illustrated.

Discussion

The individual R_{pi}-genes used in this study have been described before in the literature. The phenotypic effects of resistance based on $R_{Pi\text{-}mcd1}$ are described in Tan et al. (2008). It was then estimated that $R_{Pi\text{-}mcd1}$ causes a delay of infection of about 1–2 weeks. In this experiment though, genotypes with $R_{Pi\text{-}mcd1}$ pass the level of 50% infection on average 3 days later than R_{pi}-gene free genotypes. The shorter delay of infection by $R_{Pi\text{-}mcd1}$ in this experiment is likely caused by the relatively cold and humid weather favouring rapid late blight development in the summer of 2007.

Rauscher et al. (2006) mapped $R_{Pi\text{-}ber}$ 5.1 cM south of marker CT214 and 0.6 cM north of marker TG63. The resistance we observed in this study mapped to chromosome *10,* close to marker TG63, but 1.3 cM south of marker TG63. Although the position of marker TG63 relative to $R_{Pi\text{-}ber}$ differs in this study with the marker order shown by Rauscher et al., we do not wish to draw strong conclusions on only one recombination event. Therefore, we cannot present conclusive evidence that this resistance is a different locus to the previously reported $R_{Pi\text{-}ber}$. The difference in position of marker TG63 could be attributed to false positive/negative marker or phenotypic scoring which can make a large impact when dealing with a small population and a small number of markers. An alternative explanation could be that marker TG63 is located inside (and thus flanked by) a large cluster of R_{pi}-gene homologues. The use of one accession or another accession with different functional members of the cluster could lead to alternative mapping orders. $R_{Pi\text{-}ber}$, originating from *S. berthaultii*, was reported as a new R_{pi}-gene by Ewing et al. (2000) and Rauscher et al. (2006). According to information on internet by Dr. J. Jones, there are two R_{pi}-genes located on chromosome *10* of *S. berthaultii* named $R_{Pi\text{-}ber1}$ and $R_{Pi\text{-}ber2}$ (http://www.ayeaye.tsl.ac.uk/index.php?option=com_content&task=view&id=98&Itemid=59). They are both roughly in the same position as the previously identified $R_{Pi\text{-}ber}$ (Ewing et al. 2000; Rauscher et al. 2006). $R_{Pi\text{-}ber1}$ may be the same gene as $R_{Pi\text{-}ber}$. Because we were uncertain if the *S. berthaultii* resistance from our accession CGN 17823/PI265858) was the same locus as the previously identified $R_{Pi\text{-}ber}$, we confirmed its map position using data from a field experiment. The data of this field experiment also confirmed that the resistance conferred by $R_{Pi\text{-}ber}$ is not absolute, but can be described as strong in effect.

The objective of this research was to pyramid two functional R_{pi}-genes originating from wild potato species in a *S. tuberosum* background and to compare the resistance level of those pyramided R_{pi}-genes with the level of the single R_{pi}-genes. We combined $R_{Pi\text{-}mcd1}$ with $R_{Pi\text{-}ber}$. Both R_{pi}-genes confer incomplete resistance to *P. infestans* but greatly differ in their effect. The $R_{Pi\text{-}mcd1}$ gene offers a small but significant reduction of the infection, lasting only for a short period while the $R_{Pi\text{-}ber}$ resistance gene offers a strong effect. It took 36 days until 50% of the average leaf area was infected in plants that carry the $R_{Pi\text{-}ber}$ resistance gene. In the field trial, the plants with the $R_{Pi\text{-}ber}$ resistance gene did not confer immunity during the entire growth cycle. The failure of the resistance by the end of the growing cycle is an annually observed phenomenon, known from other trials and years using this genetic material (data not shown). This is best explained by the physiological aging of the plant. Pyramiding of $R_{Pi\text{-}mcd1}$ and $R_{Pi\text{-}ber}$ resulted in a higher level of resistance. This is not an obvious conclusion that should have been expected before starting this experiment. In view of the large effect of the $R_{Pi\text{-}ber}$ gene until late in the growing season, and in view of the short delay of infection at the beginning of the infection period we could also have expected that effect of the $R_{Pi\text{-}ber}$ gene would entirely surpass the modest effect of the $R_{Pi\text{-}mcd1}$ gene. The resistance level in the pyramid group showed an additive effect of $R_{Pi\text{-}mcd1}$ with $R_{Pi\text{-}ber}$ without clear evidence for a 'law of diminishing returns'.

In our experiment, we used a large population of full sibs. This sibling structure would on average nullify the interaction between a specific genetic background and the R_{pi}-genes. One example of such an interaction is the influence of maturity type on resistance level, irrespective of R_{pi}-gene composition. This experimental design proved to be effective, because it allowed us to compensate for maturity effects. In our experiment, the magnitude of the effect caused by the individual R_{pi}-genes was slightly obscured by variation in maturity. When resistance levels were corrected for maturity type, the difference between pyramided and the single $R_{Pi\text{-}ber}$ resistance levels stayed significant for a longer period of time: 9 days instead of 3 days.

The pyramiding of R_{pi}-genes is a valid strategy if the anticipated benefits exceed the breeding efforts. It is

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debatable if an infection delay of a couple of days is valuable. In our pyramided group, the resistance level of $R_{Pi\text{-}ber}$ was complemented by the resistance level of $R_{Pi\text{-}mcd1}$. $R_{Pi\text{-}mcd1}$ is known to be effective for a limited period. Therefore, improvement of resistance levels would be more economic when using R_{pi}-genes with stronger effects. Irrespective of the complexities of natural infection with late blight, our findings indicate that combining the R_{pi}-genes $R_{Pi\text{-}mcd1}$ and $R_{Pi\text{-}ber}$ in potato is useful. In view of the additivity of the effects of the $R_{Pi\text{-}mcd1}$ and $R_{Pi\text{-}ber}$ genes, and the lack of evidence for the 'law of diminishing returns', we recommend the development and analysis of potato clones with additionally pyramided R_{pi}-genes to investigate the hypothesis that resistance levels comparable to non-host resistance might be feasible (Heath 2001; Jeuken and Lindhout 2002).

More important than economic considerations, the strategy of pyramiding R_{pi}-genes is valued because of epidemiological and evolutionary considerations. Besides raising the level of resistance, pyramiding R_{pi}-genes might contribute to the durability of resistance. The pathogen would need double or multiple mutations to overcome the resistance. The durability of an R_{pi}-gene can be perceived as the evolutionary potential (mutation and recombination) of the plant pathogen to avoid R_{pi}-gene recognition. In that case R_{pi}-gene durability is based on the evolution of effector molecules and the indispensability of their current function. But when discussing durability of single or pyramided R_{pi}-genes, one should be aware that durability can not be predicted in advance, but can only be reflected upon by historical evaluation. To ensure a maximum lifespan of an R_{pi}-gene though, it is important that single R_{pi}-gene and pyramided plants are not deployed simultaneously. The single-gene plants would in that case provide a "stepping-stone" for pathogens to overcome each of the pyramided resistance genes (Zhao et al. 2003).

Cultivars with pyramided R_{pi}-genes can be developed by traditional and time consuming breeding or with genetic modification. Genetic modification is assumed to be a fast way to insert two or more R_{pi}-genes into an existing variety. A huge benefit is that undesirable linkage drag from the wild potato genome, causing for example an elevated glycoalkaloid content, is prevented. Most of the *R*-genes against *P. infestans* originate from crossable wild potato species. Therefore, the GM product should not be considered as transgenic but as cisgenic (Jacobsen and Schouten 2007). As long as public acceptance for genetic modification is lacking in the EU, classical introgression breeding is required. One positive aspect of the use of diploid progenitor lines is the high frequency of gene transfer of these genes to the tetraploid potato via 4X–2X crosses. On average 80% of the heterozygous R_{pi}-gene in diploids will result in tetraploid offspring with a simplex R_{pi}-gene due to First Division Restitution of the unreduced male-gametes (Hermsen 1984). Molecular markers can facilitate the selection of superior descendants by tracing the R_{pi}-genes in the breeding process and removing linkage drag efficiently.

In this paper, we have tested the hypothesis whether or not pyramiding is a valid strategy. There was no *a* priori expectation on the added value of the weaker $R_{Pi\text{-}mcd1}$ locus in the presence of the stronger $R_{Pi\text{-}ber}$ locus. Our results suggest that pyramiding can result in an additive effect of the individual genes on the level of resistance. In our opinion, potato breeding can benefit from combining individual R_{pi}-genes.

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Polyphenol Oxidase in Potato[1]

A Multigene Family That Exhibits Differential Expression Patterns

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Polyphenol oxidase (PPO) activity in potato (*Solanum tuberosum*) plants was high in stolons, tubers, roots, and flowers but low in leaves and stems. PPO activity per tuber continued to increase throughout tuber development but was highest on a fresh weight basis in developing tubers. PPO activity was greatest at the tuber exterior, including the skin and cortex tissue 1 to 2 mm beneath the skin. Flowers had high PPO activity throughout development, particularly in the anthers and ovary. Five distinct cDNA clones encoding PPO were isolated from developing tuber RNA. POT32 was the major form expressed in tubers and was found in all parts of the tuber and at all stages of tuber development. It was also expressed in roots but not in photosynthetic tissues. POT33 was expressed in tubers but mainly in the tissue near the skin. POT72 was detected in roots and at low levels in developing tubers. NOR333 was identical with the F2 PPO clone previously isolated from potato leaves (M.D. Hunt, N.T. Eannetta, Y. Haifeng, S.M. Newman, J.C. Steffens [1993] Plant Mol Biol 21: 59–68) and was detected in young leaves and in tissue near the tuber skin but was highly expressed in flowers. The results indicate that PPO is present as a small multigene family in potato and that each gene has a specific temporal and spatial pattern of expression.

PPO is the major cause of enzymic browning in higher plants (Vaughn et al., 1988). PPO catalyzes the conversion of monophenols to *o*-diphenols and *o*-dihydroxyphenols to *o*-quinones. The quinone products can then polymerize and react with amino acid groups of cellular proteins, resulting in black or brown pigment deposits. Such damage causes considerable economic and nutritional loss in the commercial production of fruit and vegetables (Vamos-Vigyazo, 1981). PPO is localized in plastids, and although membrane associated, it is not an integral membrane protein (Vaughn et al., 1988). In vivo, the phenolic substrates of PPO are localized in the vacuole and the browning reaction only occurs as a result of tissue damage leading to a loss of this subcellular compartmentation.

Various physiological roles have been proposed for PPO (Vaughn et al., 1988). Because of the plastidic location of PPO, it was postulated to play a role in the photosynthetic reactions of chloroplasts, but it is now more widely accepted that PPO is probably involved in defense against invading pathogens or insect pests (Mayer, 1987; Steffens et al., 1994). Little information is available about the regulation of PPO gene expression in plants, but a number of PPO genes from different plant species have now been isolated and characterized. All encode peptides of approximately 67 kD that give rise to mature proteins of approximately 60 kD following cleavage of a transit peptide from the N terminus during import into the plastids (Cary et al., 1992; Robinson and Dry, 1992; Shahar et al., 1992; Hunt et al., 1993; Newman et al., 1993; Dry and Robinson, 1994). In some species PPO genes are present as multigene families (Cary et al., 1992; Newman et al., 1993), whereas in others only a single PPO gene has been identified (Dry and Robinson, 1994). All of the PPO genes characterized thus far are nuclear encoded (Cary et al., 1992; Robinson and Dry, 1992; Shahar et al., 1992; Hunt et al., 1993; Newman et al., 1993). PPO contains copper, which is essential for its activity (Delhaize et al., 1985), and the greatest sequence conservation within and between species is around the His residues, which are postulated to bind the prosthetic copper atoms to the protein (Dry and Robinson, 1994).

Most of the PPO genes characterized so far have been isolated from photosynthetic tissues, where the plastids are in the form of chloroplasts, but PPO is also present in nonphotosynthetic tissues. Enzymic browning mediated by PPO is particularly apparent in potato (*Solanum tuberosum*) tubers (Matheis, 1987; Corsini et al., 1992), where the enzyme is localized within amyloplasts of the tuber cells (Czaninski and Catesson, 1974). Two PPO genes previously isolated from potato by Hunt et al. (1993) were found to be expressed in leaves, flowers, roots, and petioles, but no expression was detected in tubers. We now report the isolation and characterization of novel PPO genes from potato tubers. There appear to be a number of different PPO genes in potato plants, each with specific spatial and temporal patterns of expression.

MATERIALS AND METHODS

Plant Material

Potato (*Solanum tuberosum* cv Norchip, cv Saturna, and cv Atlantic) plants and tubers were obtained from commercial fields in South Australia at 3- to 4-week intervals.

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Abbreviations: PPO, polyphenol oxidase; RACE, rapid amplification of cDNA ends.

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PPO Activity

Tissues were diced prior to extraction and added to 5 volumes of grinding buffer (25 mM Mes, 10 mM ascorbate, pH 6.0). Enzyme extracts were prepared by homogenizing the tissue with a Polytron blender (Kinematica AG, Littau, Switzerland). Cell debris was removed by filtration through Miracloth (Calbiochem). PPO activity of extracts was measured as the initial rate of oxygen uptake at 25°C in 50 mM sodium phosphate, pH 6.0. The reaction was initiated by the addition of the substrate 4-methyl catechol to a final concentration of 2 mM. One unit of activity was defined as that which catalyzes the consumption of 1 μmol of oxygen per minute under the assay conditions.

Nucleic Acid Manipulations

Total RNA was extracted from potato tissues according to the method of Logemann et al. (1987). mRNA was isolated using the PolyATract system (Promega). First-strand cDNA was synthesized using avian myeloblastosis virus reverse transcriptase and oligo(dT) primer, and partial PPO cDNAs were amplified by PCR using 3′ RACE (Frohman et al., 1989) with degenerate oligonucleotide primers. PCR products were end filled with Klenow fragment and cloned into pBluescript SK+ (Stratagene). The 5′ ends of PPO cDNAs were obtained by 5′ RACE (Frohman et al., 1989) with nested antisense primers and cloned as described above. DNA sequencing was carried out according to the method of Sanger et al. (1977) with T7 DNA polymerase or *Taq* DNA polymerase. DNA was sequenced on both strands. Northern analysis was carried out as described previously (Robinson and Dry, 1992). When different probes were used, they were of comparable specific activity and blots were exposed to x-ray film for equal times.

Sequence Analysis

DNA and protein sequences were analyzed using the PC/Gene sequence analysis package (Intelligenetics, Inc., Mountain View, CA) and the GCG package (Genetics Computer Group, 1991).

RESULTS

PPO Activity in Potato Tissues

The PPO activity of various potato tissues (cv Atlantic) was determined and the results are shown in Table I. The highest PPO activity was in stolons, tuber buds, and roots, with moderate levels in young leaves and mature tubers and low activity in mature leaves and stems. PPO activity within flowers was high in the reproductive tissues, i.e. the ovary (including the stigma and style) and the anthers, but low in the petals and sepals (Table I). The PPO activity throughout leaf and flower development was further investigated, and the data are shown in Table II. PPO activity in leaves was highest in the youngest stage assayed (<1 cm long) and declined to virtually undetectable levels in fully expanded leaves (5–6 cm long). PPO activity was high throughout flower development with a maximum in large, unopened, not fully developed flowers. Activity in opened flowers decreased by approximately 50% but was still relatively high.

Table I. *PPO activity in different potato tissues (cv Atlantic)*

Tissue	PPO Activity
	units g^{-1} fresh wt
Stems	2
Mature leaves	7
Young leaves	17
Roots	144
Stolons	221
Tuber buds	236
Mature tubers	45
Petals	10
Sepals	17
Anthers	81
Ovary, stigma, and style	83

PPO activity in tubers of varying sizes, assumed to reflect tuber age or stage of development, was determined and the results are shown in Figure 1, A and B. The magnitude of PPO activity varied with the cultivar of potato assayed, being 3 to 4 times higher in cv Saturna than in cv Atlantic. In both cultivars PPO activity per tuber accumulated almost linearly throughout tuber development, whereas PPO activity per gram fresh weight of tuber was high in young tubers and declined to a level that remained constant throughout subsequent tuber growth. The distribution of PPO activity within the "peel" and "cortex" fractions is shown in Figure 1, C and D. "Peel" in this instance comprised the outer skin and approximately 1 mm of tuber cortex, which was removed with a common vegetable peeler. The remaining tissue was designated "cortex." On a fresh weight basis, PPO activity of the peel fraction was higher than that of tuber cortex at all stages of tuber development and declined less than the activity in the cortex with increasing tuber size (Fig. 1D). The peel contributed only a small proportion of the weight of the tuber and hence only a small amount of the total PPO activity of the tuber (Fig. 1C). The relative contributions of the peel and cortex fractions to the PPO activity of tubers of cv Saturna were essentially the same as for cv Atlantic (data not shown).

To obtain a finer resolution of the distribution of PPO activity within the tuber, we determined the activity profile along the longitudinal axis of developing tubers. The peel fraction was further subdivided into the skin and the outer cortex (defined as the exterior 1-mm layer of cortex), and the remainder of the cortex was sectioned in 2-mm-thick slices. Each of these fractions was assayed for PPO activity and Figure 2 illustrates that, although the skin contained a significant amount of PPO activity, the highest activity was recorded in the subepidermal or outer cortex layer. PPO activity then decreased to a level that was constant through to the center of the tuber.

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Table II. *PPO activity throughout potato leaf and flower development (cv Atlantic)*

Tissue	PPO Activity
	units g^{-1} fresh wt
Leaf stage	
1 (<1 cm)	57
2 (1–2 cm)	33
3 (2–3 cm)	21
4 (5–6 cm)	3
Flower stage	
1 (small buds)	112
2 (medium buds)	128
3 (large buds, not fully developed)	163
4 (large buds, fully developed)	148
5 (open flowers)	77

Isolation and Characterization of PPO Genes from Potato Tubers

Oligonucleotide primers complimentary to conserved regions around the putative copper-binding residues of PPO amino acid sequences from plants (Dry and Robinson, 1994) were synthesized. These primers were used to amplify PPO cDNAs using the 3′ RACE technique (Frohman et al., 1989).

Five distinct cDNA clones, designated POT32, POT33, POT41, POT72, and NOR333, were isolated from young developing tubers (<1 g, cv Norchip) using this approach. The 5′ ends of POT32, POT33, and POT41 were obtained using the 5′ RACE technique with nested antisense primers designed from the original cDNA sequences. POT32 and POT41 are 95% identical at the nucleic acid level. NOR333 was virtually identical (1 bp difference over 1100 bp) with the P2 PPO gene isolated from potato leaves (Hunt et al., 1993). The four clones POT32, POT41, POT33, and POT72 are all 70 to 72% identical with P2. POT32 (and POT41), POT33, and POT72 all share approximately 82% identity with each other.

Analysis of the deduced amino acid sequence of the full-length clones of POT32 and POT33 and the partial sequences of POT41, POT72, and NOR333 revealed considerable similarity to other plant PPO sequences, particularly around the highly conserved copper-binding sites (Steffens et al., 1994), thus confirming their identity as PPO clones.

POT32 is 1958 bp in length and contains an open reading frame of 1791 bp encoding a 597-amino acid peptide of 66.9 kD. POT33 is 1943 bp long, contains an open reading frame of 1797 bp, and encodes a 599-amino acid peptide of 67.5 kD. Amplification of the coding sequences of POT32 and POT33 by PCR from either tuber cDNA or genomic DNA generated fragments identical in size with the cDNA clones, indicating that neither of these genes possesses introns (data not shown). The predicted protein sequences of POT32 and POT33 (Fig. 3) possess putative plastid transit peptide sequences of 89 amino acids. The transit sequences are rich in the hydroxylated amino acids Ser and Thr and the small, hydrophobic amino acids Ala and Val and possess a net positive charge, all characteristics of transit peptides (Keegstra et al., 1989). The transit peptide is known to be cleaved from other PPO proteins to give rise to the mature protein (Sommer et al., 1994). PPO was purified from the cortex of mature potato tubers and the N-terminal amino acid sequence of the 60-kD protein was

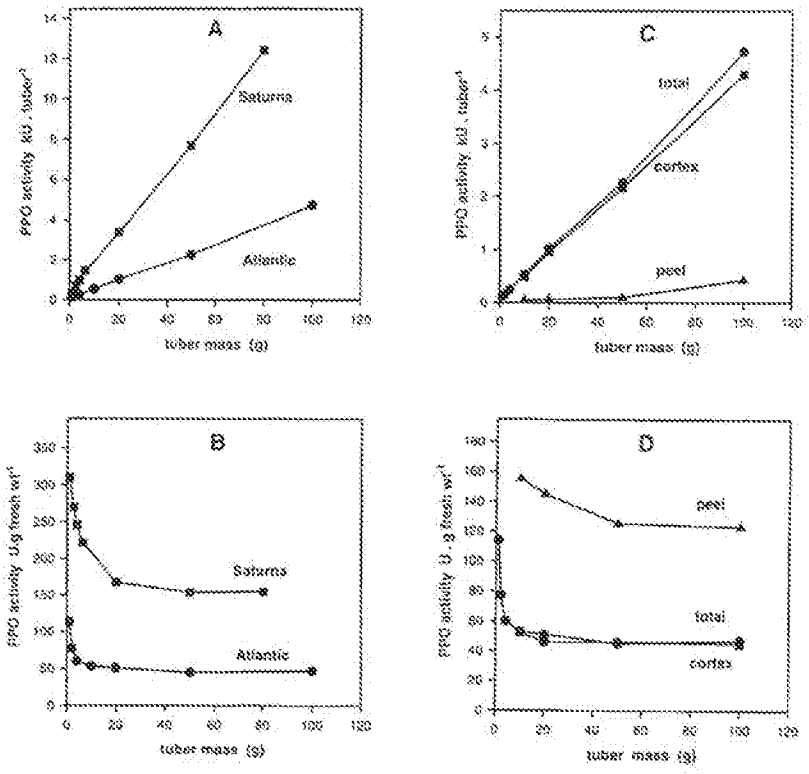


Figure 1. PPO activity in developing potato tubers. A, Accumulation of PPO activity per tuber during growth in cv Atlantic (●) and Saturna (■). B, PPO activity g^{-1} fresh weight (wt) of tuber in cv Atlantic (●) and Saturna (■). C, PPO activity in peel and cortex per tuber of cv Atlantic. ▲, Peel (skin plus approximately 1 mm of cortex); ■, remainder of the tuber; ●, total. D, PPO activity (g^{-1} fresh weight of tuber) in peel (▲), cortex (■), and total tuber (●) of cv Atlantic. kU, Kilounits; U, units.

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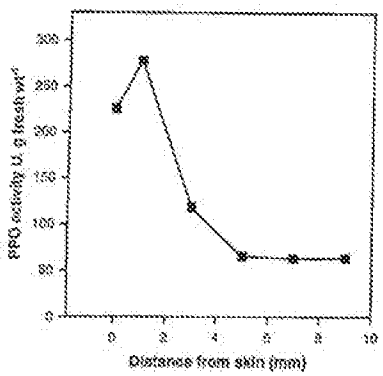


Figure 2. Distribution of PPO activity in a longitudinal section through a tuber. A 70-g tuber (cv Atlantic) was sectioned along a longitudinal axis at 2-mm intervals, and the PPO activity of each section was measured. U, Units; wt, weight.

determined by automated sequencing (Thygesen et al., 1994). This N-terminal sequence from the purified tuber PPO protein (APAPFPDLSS) was identical with the sequence of the mature protein predicted from the clone POT32, confirming that this sequence does indeed encode a potato tuber PPO.

An alignment of the predicted protein sequences of POT32 and POT33 with that of the P2 PPO sequence isolated from potato leaves (Hunt et al., 1993) is shown in Figure 3. The relationship of POT32 and POT33 to each other and to PPOs isolated from other species is shown in Table III. The homology between the PPO protein sequences of potato and tomato (Newman et al., 1993) is very high, and there is an obvious similarity between the species. POT32 and tomato PPO D, POT33 and tomato PPO B, and P2 (=NOR333) and tomato PPO E share greater identity to their interspecific homolog than to the other intraspecific PPOs (Table III).

Spatial Expression of PPO Genes in Potato Tissues

POT32, POT33, POT72, and NOR333 were used to probe RNA isolated from a range of potato tissues, and the results are shown in Figure 4. The unique pattern of expression evident for each of these clones indicates that they are differentially expressed. POT32 was strongly expressed in the inner and outer cortex of tubers and in roots. There was some expression in tuber skin, but POT32 mRNA was not detected in leaves or stems. POT33 mRNA was most highly expressed in the outer cortex of tubers, and POT72 was expressed predominantly in roots with a small amount of message detectable in the inner and outer cortex of tubers.

Probing of potato tissues with the clone NOR333 elicited very strong hybridization to RNA from unopened flower buds, and there was also considerable hybridization to RNA from young leaves but none from mature leaves (Fig. 4). NOR333 also hybridized to RNA isolated from potato tubers (Figs. 4 and 5). However, this hybridization was confined to RNA from the "outer cortex" fraction of the tuber and elicited very poor or no hybridization to RNA from either the skin or inner cortex (Fig. 4). Southern blot experiments with a dilution series of the PPO cDNA clones indicated that there was <5% cross-hybridization between clones at the same stringency used for the northern blots (data not shown). Thus, except for the flower RNA sample, the possibility of hybridization signals resulting from cross-hybridization to other PPO transcripts seems unlikely. The level of NOR333 transcript detected in flowers was so great that faint signals elicited by POT32 and POT33 in flowers (Fig. 4) may be attributed to cross-hybridization to NOR333 transcripts.

Temporal Expression of PPO Genes in Potato Tissues

POT32, POT33, POT72, and NOR333 were used to probe RNA isolated from a developmental series of tubers of cv Saturna (the same as that assayed for PPO activity shown in Fig. 1). RNA was isolated from the whole tuber tissue. The four genes all exhibited a similar trend of expression, being highest in young tubers and lower in older ones; however, the absolute levels of transcript varied considerably (Fig. 5). The greatest hybridization signal was elicited by POT32, and its level was highest in small tubers and lower in larger, older tubers, with transcript still detected in 80 g of tubers. POT32 was the predominant PPO message expressed in these older tubers. NOR333 expression was high in young tubers but decreased markedly in older tubers. The expression of POT33 in tubers was lower than that of either POT32 or NOR333, but it was higher in younger tubers relative to older tubers. POT72 was barely detectable, even in young tubers.



Figure 3. Alignment of potato PPO protein sequences. The deduced PPO protein sequences of POT32 and POT33 were aligned with the P2 PPO protein (Hunt et al., 1993). Identical residues in all three proteins are indicated by asterisks (*); the N terminus of the mature protein is indicated by an arrowhead; the copper-binding regions, CuA and CuB, are boxed and shaded; the His residues predicted to be copper-binding ligands are shown in bold type.

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Table III. *Homology of PPO protein sequences*

The percentage of identities of POT32 and POT33 with PPO protein sequences from various plant species were calculated using the "Distances" program of the GCG package. POTP2, Potato leaf (P2, Hunt et al., 1993); Tom B, tomato PPO B; Tom D, tomato PPO D; Tom E, tomato PPO E (Newman et al., 1993); Bean, bean leaf (Cary et al., 1992); Apple, from Boss et al. (1995); Grape, grape berry (Dry and Robinson, 1994). Note that POTP2 is equivalent to NOR333. High interspecific identity of potato and tomato PPOs is indicated in bold type.

	POT32	POT33	POTP2	Tom B	Tom D	Tom E	Bean	Apple	Grape
POT32	100	85.1	79.1	86.1	**93.6**	79.1	54.4	56.3	54.2
POT33		100	80.1	**95.8**	84.4	80.3	53.7	55.7	56.5
POTP2 (=NOR333)			100	80.0	76.2	**94.6**	56.1	56.1	56.3
Tom B				100	85.6	80.1	55.0	56.0	57.1
Tom D					100	76.8	53.6	55.3	53.3
Tom E						100	56.6	56.2	57.6
Bean							100	71.3	63.9
Apple								100	66.5
Grape									100

A more detailed examination of the expression of NOR333 during leaf and flower development is shown in Figure 6. Expression of NOR333 was evident in the youngest leaves (L1), barely detectable in the L2 stage, and not detected in the older leaves. There was strong expression of NOR333 in the four unopened flower stages, F1 through F4. The level of NOR333 mRNA increased with flower age, reaching a maximum at stage F3, declined somewhat in stage F4, and was virtually absent in fully opened flowers, stage F5. Probing of RNA from the reproductive parts of F3-stage flowers indicated that the greatest expression of NOR333 was in the anthers. There was also very high expression in the ovary, considerable expression in petals, and virtually none in sepals.

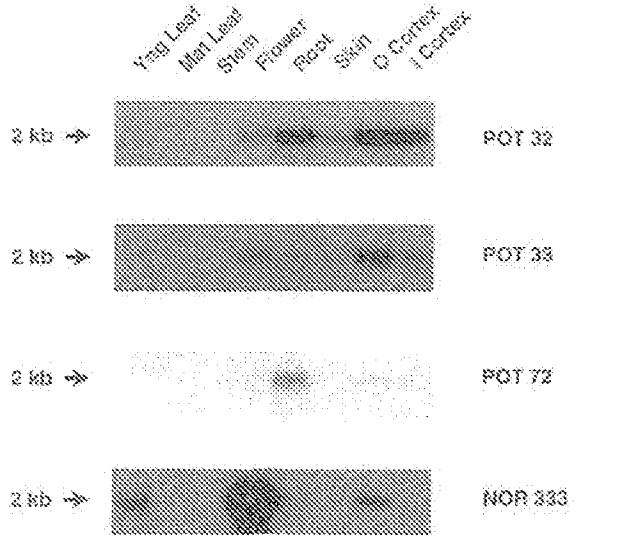


Figure 6. Tissue-specific expression of PPO genes in potato. Total RNA was isolated from potato tissues (cv Atlantic): young leaves (Yng Leaf), mature leaves (Mat Leaf), stems, unopened flower buds (flowers), roots, tuber skin (Skin), the outer cortex of tubers (the tissue 1–2 mm below the skin; O Cortex), and the remainder of the tuber tissue (inner cortex; I Cortex). RNA was extracted from 20-g tubers. RNA (20 μg) from each tissue was probed with POT32, POT33, POT72, or NOR333. The position of the 2-kb RNA marker is indicated beside each panel.

DISCUSSION

We have isolated five distinct cDNA clones encoding PPO from potato tubers, and these clones share considerable homology with other PPO genes, especially those previously isolated from potato leaves (Hunt et al., 1993) and tomato (Shahar et al., 1992; Newman et al., 1993). POT32, POT33, POT41, and POT72 are distinct from the two PPO genes, P1 and P2, previously isolated from potato leaves (Hunt et al., 1993). Somewhat surprisingly, however, NOR333, which was also isolated from tuber cDNA, was found to be identical with the P2 leaf gene.

PPO forms a multigene family of seven distinct genes in tomato (Newman et al., 1993), and Cary et al. (1992) also reported the presence of at least three PPO genes in *Vicia faba*. We now present evidence for a multigene family of at least six PPO genes in potato: the four novel cDNAs reported here and the two genes described by Hunt et al. (1993). The considerable homology of the P1 and P2 and POT32 and POT41 gene pairs of potato and the fact that potato is a tetraploid suggest that these pairs may represent homologs from the different chromosome sets. Consistent with other reports of PPO gene structure (Shahar et al., 1992; Newman et al., 1993; Dry and Robinson, 1994), we found no evidence that either POT32 or POT33 contains introns. This raises the possibility that PPO was originally a chloroplast-encoded gene that was "sequestered" to the nucleus.

The PPO activity in potato tissues was mirrored by PPO gene expression, and this correlation suggests that the control of PPO levels in the potato plant is mediated at the level of transcription. Thus PPO activity was high in developing flowers, developing leaves, and developing tubers and roots (Tables I and II), and the highest levels of PPO mRNA were found in developing flowers and developing tubers and in young leaves (Figs. 4–6). These data are consistent with previous studies in which PPO gene expression has generally been greatest in developing tissues and meristematic regions, with expression declining as development proceeds (Cary et al., 1992; Hunt et al., 1993; Dry and Robinson, 1994; Boss et al., 1995). It is interesting that in the case of tubers PPO activity and gene expression

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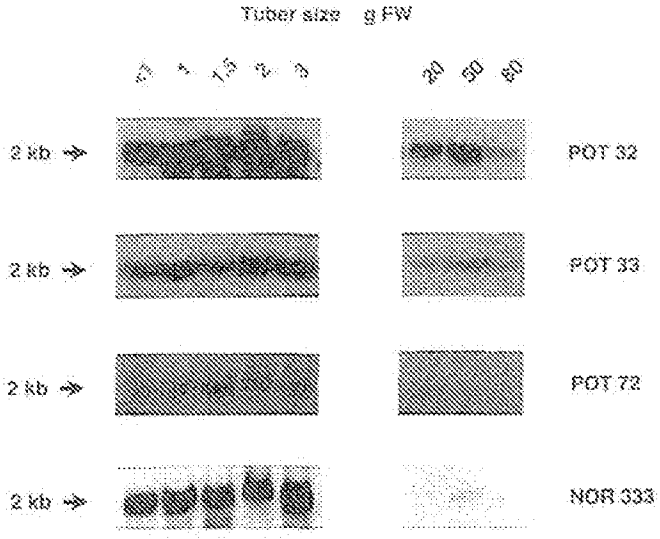


Figure 5. The temporal expression of PPO genes in potato tubers. Total RNA was isolated from whole tubers (cv Atlantic) of varying sizes. Tubers were dug early and later in plant development, and this is indicated by the two separate panels. RNA (20 μg) was probed with POT32, POT33, POT72, or NOR333. The position of the 2-kb RNA marker is indicated beside each panel. FW, Fresh weight.

are maintained beyond early development and throughout tuber development and growth (Figs. 1 and 5).

The control of PPO gene expression in tubers is complex, with the different genes exhibiting distinct patterns of spatial and temporal expression. POT32 and POT33 were expressed predominantly in tubers, with POT32 being the major form detected in all parts of the tuber and throughout tuber development. The identity of the predicted mature N terminus of POT32 with that of PPO purified from tuber cortex provided further verification that POT32 is probably the major mRNA species expressed in tuber cortex. However, given the high degree of homology of POT41 with POT32, we cannot rule out the possibility that POT41 is also highly expressed in these tissues. POT32 was also the major form expressed in roots. POT33 expression was confined to the tuber, where it was most highly expressed in the outer cortex. It was also expressed throughout tuber development but to a much lesser extent than was POT32. The expression of POT72 was barely detectable in tubers and seemed to be almost exclusively expressed in roots but at a much lower level than POT32. POT32, POT33, and POT72 might therefore be considered, if not "tuber genes," at least genes expressed in nonphotosynthetic tissues.

Given that Hunt et al. (1993) detected expression of the P1 gene (95% identical to NOR333) in potato leaves but not in tubers, we were surprised to detect strong expression of NOR333 in tubers. In contrast to POT32 and POT33, NOR333 was expressed only early in tuber development, and its expression was confined to the subepidermal 1 mm, which we termed the outer cortex. We presume that the inner cortex, where there was no expression of NOR333, was analogous to the "mature tuber periderm" probed by Hunt et al. (1993), thus providing an explanation for their failure to detect expression [illegible]

NOR333 expression in leaves followed the same trend observed for PPO activity: it was highest in very young leaves and declined markedly with leaf age. The expression of NOR333 in young flowers was far in excess of that detected for any of the genes in the tissues tested, perhaps justifying a description as a "floral" rather than a "leaf" gene. Although expression of NOR333 was high in flower buds, it was virtually absent in opened flowers, consistent with the data of Hunt et al. (1993), who detected very low expression of PPO P1 in mature potato flowers. Within the flower the greatest expression was detected in anthers, ovary, and petals, and expression was very low in sepals. This is also consistent with the high PPO activity present in the reproductive tissues. Shahar et al. (1992) investigated the expression of a PPO gene, cP2/A1, in various tomato tissues (cP2/A1 shares considerable homology with NOR333). Expression of cP2/A1 was detected at high levels in the reproductive tissues of flowers 3 to 4 d prior to anthesis and in vegetative meristems. No mRNA expression was detected in the sepals of mature flowers or in tomato fruit. Although it is not possible to make a direct comparison of the developmental stage of the tomato flowers used by Shahar et al. (1992) with the potato flowers assayed in this study, a consistent pattern of PPO expression appears to be evident for the two species. It is possible that the profound reduction of PPO mRNA observed in opened flowers reflects a general shutdown of transcription upon flower opening.

Newman et al. (1993) classified the six PPO genes of tomato into three classes on the basis of restriction fragment length polymorphism mapping. There is extensive homology between individual genes of potato and tomato (>92% identity, Table III) and on the basis of sequence

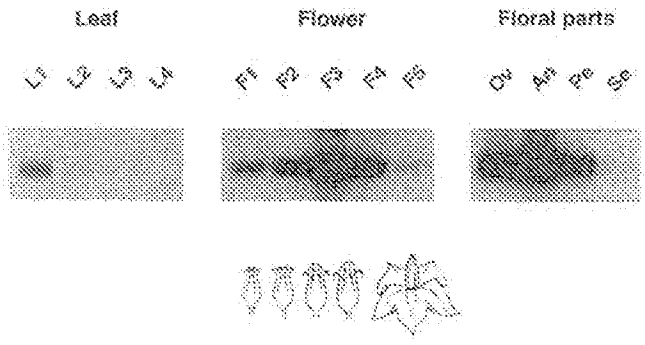


Figure 6. Temporal and spatial expression of the PPO gene NOR333 in potato leaves and flowers. Total RNA was isolated from leaves and flowers of different ages and from floral parts. L1, <1 cm long with yellow-green apices; L2, 1 to 2 cm long; L3, 2 to 5 cm long, green, and still expanding; L4, 5 to 8 cm long, dark green, and fully expanded. F1, Small buds, sepals longer than petals, and petals were green; F2, medium size buds, sepals the same length as petals, and petals still green; F3, buds unopened and still developing, petals longer than sepals, and petals were green-white; F4, buds unopened but fully developed and petals were white; F5, opened flowers. The flower stages are depicted below the panel. Ov, Ovary, stigma, and style; An, anthers; Pe, petals; Se, sepals and pedicel. RNA (20 μg) from each tissue was probed with NOR333. The hybridizing bands

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homology each of the genes we have isolated falls into one of these three classes, i.e. class I, POT33 and POT72 (data not shown); class II, POT32 and POT41; and class III, NOR333 (P2) and P1 (Hunt et al., 1993). It is tempting to attribute some functional significance to this conservation of classes of PPO genes between potato and tomato, and an obvious candidate is tissue specificity. Class III genes appear to be expressed in photosynthetic tissues in tomato (Shahar et al., 1992; Newman et al., 1993) and in potato (Hunt et al., 1993; this study). In contrast POT32, POT33, and POT72 are all expressed in nonphotosynthetic tissues of potato. By analogy, therefore, the class I and class II genomic PPO genes of tomato (Newman et al., 1993) would also be predicted to be expressed in nonphotosynthetic tissues, although this remains to be determined. In view of the complex patterns of expression of PPO genes in potato, it would be interesting to isolate and analyze the promoters responsible for regulating the tissue-specific expression of this multigene family, particularly in tubers.

A definite biological function is yet to be attributed to PPO (Vaughn et al., 1988), but it seems likely that it has a role in plant defense against predation (Steffens et al., 1990) or pathogen attack (Mayer, 1987). The maintenance of high PPO activity and gene expression throughout tuber development and the localization of the highest levels at the tuber exterior are consistent with a role in the protection of this storage and propagative organ from predation or infection. High levels in young, developing tissues and in the functional parts of flowers also support such a role. Preliminary experiments indicate that PPO activity and the expression of POT32, POT33, POT72, and NOR333 can be induced to high levels in tubers following wounding (S.P. Robinson and P.W. Thygesen, unpublished results), providing further support for the notion that PPO is an enzyme involved in defense against predation or infection.

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Occurrence of the *R1* allele conferring resistance to late blight in potato R-gene differentials and commercial cultivars

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A total of 23 Scottish and 14 Dutch potato R-gene differentials as well as five Austrian, two Dutch and two German commercial potato cultivars were screened for the *R1* allele conferring resistance to *Phytophthora infestans* carrying *Avr1*, via PCR amplification and sequencing. A single 1400 bp fragment with complete sequence identity to the corresponding part of the *R1* allele, was obtained from genomic DNA of all potato R-gene differential clones whose denomination indicates *R1* or a combination of *R1* and other major resistance factors. The *R1* allele was detected, as expected, in all these clones. This fragment also occurred in one Austrian and one German cultivar. Unexpectedly, the same *R1* allele also was detected within all R5, R6 and R9 differentials.

Keywords: international R-gene differential set, *Phytophthora infestans*, *R1* allele

Introduction

The potato-late blight pathogen, *Phytophthora infestans*, forms physiologic races that are able to circumvent resistance determined by specific *R* genes of *Solanum*. Accordingly, the solanaceous hosts of *P. infestans*, including potato, tomato, pepper, pepino dulce and many other species, have evolved multiple *R* genes for race-specific resistance to late blight. Major *R* genes of plants have often been detected in segregating progenies by their highly heritable resistance phenotype. The total number of *R* genes for resistance to late blight in *Solanum* spp. is unknown; they may exceed considerably those represented by the current international set of eleven R-gene differentials (Black *et al.*, 1953; Malcolmson & Black, 1966).

The *R1* gene, characterized by Ballvora *et al.* (2002), confers resistance to strains of *P. infestans* carrying *Avr1* (Van der Lee *et al.*, 2001). *R1* was introduced into the common potato (*S. tuberosum*) from the wild hexaploid, *S. demissum* via cross hybridization and selection for the R1 phenotype. This utilization of potato genetic resources commenced by potato researchers in the 19th century unfortunately has not been fully documented. It is not known whether *R1* was introduced into potato breeding stocks from only one or several different genetic resources. Early accounts (Schick, 1932) refer to a limited number of genetic resources from *S. demissum* and *S.* × *edinense*, its natural hybrid with the cultivated potato that had been used for hybridization experiments by Broili. The German plant pathologist and breeder K.O. Müller described the use of clonal hybrid selections that he labelled 'Ef-Stamm' ('Edinense fraglich'; compare Müller, 1951) referring to their possible taxonomic identity with *S.* × *edinense*. This and other clonal samples, perhaps of a common descent, had been distributed among several potato breeders across the world before 1930.

In 1953, a consortium of potato breeders and pathologists established a set of potato R-gene differentials that would allow for the detection of discriminant avirulence phenotypes (Black *et al.*, 1953). This set comprised clonal selections made individually by W. Black of Scotland, by C. Mastenbroek, the Netherlands, and by W.R. Mills and L.C. Peterson, USA. Other workers at other institutions even had developed individual sets of potato differential stocks maintained as botanical seed (Schick & Schick, 1959). While none of these sets represented all *R* genes and combinations of *R* genes known at that time (by their phenotype), there were several potato clones of possibly diverse origin representing one single factor. This arose as these pioneering late blight researchers maintained differential isolates of *P. infestans* to assay the resistance phenotype of potato segregants containing unknown factors for race-specific resistance. For example, the *P. infestans* isolates breaking R1 resistance were capable of causing disease on the clonal selections namely, 1085(6) (Black's differential), 43154·5 (Mastenbroek), and Essex (Mills and Peterson), all of which attained the status (by their phenotype) of international R1 differentials (Black *et al.*, 1953).

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The international set of differentials (Black *et al.*, 1953) has been maintained clonally and was amended until the late 1960 s (Eide *et al.*, 1959; Black & Malcolmson, 1965; Malcolmson & Black, 1966; Malcolmson, 1969). It comprises clones for the detection of 11 single late blight resistance factors (classified as R1–R11) originating from *S. demissum*.

Although the *R1* gene is overcome by the majority of present-day *P. infestans* populations (Ross 1986; for examples compare Deahl *et al.*, 1991; Andrivon *et al.*, 1994; Drenth *et al.*, 1994; Forbes *et al.*, 1997; Dorrance *et al.*, 1999; Hermansen *et al.*, 2000; Elansky *et al.*, 2001), it has been of interest as a model to plant geneticists. Besides its most obvious role of producing race-specific resistance, it has been suspected to play a role in quantitative field resistance (Darsow *et al.*, 1987; Stewart *et al.*, 2003). Both race-specific and quantitative resistance in the field may, in part, employ the same mechanisms; the basic difference could be the rate and intensity of expression of resistance pathways (Ordoñez *et al.*, 1998; Gebhardt & Valkonen, 2001). The *R1* gene belongs to a large group of structurally related *R1*-like *R* genes and *R* gene analogues whose members appear to be frequent in *Solanum* spp. (Trognitz & Trognitz, 2005).

To assess the power of selectivity of the *R1*-specific PCR primers proposed by Ballvora *et al.* (2002) to tag unequivocally the *R1* allele, these primers have been applied to potato R-gene differential clones and several contemporary and older potato cultivars that are used in breeding. Whether the R1 phenotype of the respective potato R-gene differential clones is reliably associated with the occurrence of the *R1* allele was also determined.

Materials and methods

Plant material

Clones of two potato differential sets (Table 1) were included. The Scottish set of Black's differentials was kindly provided by Dr. H. Stewart, SCRI, Dundee, UK. An incomplete Dutch set has been maintained at the International Potato Centre as described in Trognitz (1998). A copy of this set was provided as stocks *in vitro* by Dr. K. Schüler, Institute of Plant Genetics and Crop Plant Research, External Branch North, Gross Lüsewitz, Germany. In addition, samples of genomic DNA of the Scottish differentials R5, R6, and R9 were kindly provided by Dr. Renata Lebecka, IHAR, Mlochow, Poland). Commercial seed tubers of Austrian, Dutch and German cultivars (Table 1) were obtained from Niederösterreichische Saatbau, Meires, Austria. Plants were maintained pathogen-free *in vitro* and grown in pots in the glasshouse at ARC-sr, Seibersdorf, Austria, according to standard procedures.

Molecular biological procedures

Total DNA was isolated from different plants grown *in vitro* or glasshouse following the instructions of the supplier (DNeasy DNA extraction kit). The *R1*-specific PCR primers 76-2sf2 and 76-2SR identified on *R1* cDNA by Ballvora *et al.* (2002) were applied as indicated by these authors, and ethidium bromide-stained PCR products were visualized on 1%-agarose gels.

For sequencing of DNA fragments, PCR products were cleaned on Sephadex columns; 1 μL of PCR product was mixed with 2 μL BigDye (V 3) reagent (Applied Biosystems), 2 μL buffer, and 0·5 μM *R1*-specific forward or reverse primer. Following the sequence reaction, the cleaned product was mixed with 10 μL HIDI (Applied Biosystems) for processing in an ABI 3100 Genetic Analyser (Applied Biosystems). Forward and reverse sequences were assembled and analysed in Sequencher software (GeneCode).

Detection of plant resistance phenotype

Detached leaflet assays were performed following the procedure of Trognitz (1998). Lateral leaflets were collected from fully expanded top leaves of adult plants. One Petri dish containing four leaflets that had been collected from at least two different clonal plants per genotype represented an experimental unit. A paper towel was folded to fit in the bottom of a Petri dish and wetted to serve as a moisture reservoir. Sporangial inoculum was prepared from infected potato tuber slices (cv. Naglerner Kipfler) and it was applied with a hand-held pump sprayer at a concentration of 20 000 sporangia/mL. The samples were incubated at 18°C for 4–8 days until symptoms were fully developed. Percentage total leaflet area diseased and degree of sporulation were assessed. For all genotypes, tests were repeated on a minimum of two dates. For the inoculations, a 'race 0' isolate of *P. infestans* (provided by W. Flier, Plant Research International, Wageningen, The Netherlands) avirulent on all 11 R-gene differentials but pathogenic on the universal suscept, r0 was used.

Results and discussion

The primers reliably amplified one fragment of the 1400 bp size expected for *R1* (Ballvora *et al.*, 2002; Gebhardt *et al.*, 2004) from 16 R-gene differential clones and two cultivars (Table 1, Fig. 1). The sequences of all fragments proved fully identical with the corresponding part of the *R1* allele at the *R1* locus (accession No. AF447489), regardless of the potato cultivar analysed.

Detached leaflet tests with race 0 gave a resistance reaction on differentials carrying R1–R11 and combinations thereof and susceptibility on the universal suscept. Two of the nine commercial cultivars genotyped were tested with race 0 and of these, Evita was susceptible and this is in accordance with the absence of the *R1* fragment. Linzer Delikatess, also was susceptible in the assays, although it carries the *R1* allele. This suggests that *R1* of Linzer Delikatess either may be suppressed by an unknown intervening genetic element (El-Kharbotli *et al.*, 1996; Ordoñez *et al.*, 1997; Trognitz, 1998), or that an additional unknown, synergistic element required for the function of

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Table 1 Potato cultivars and late blight R-gene differentials, tested for presence of the *R1* allele and for resistance to a race 0 isolate of *Phytophthora infestans*

Country of origin	R-gene differential	Clone/cultivar	*R1* fragment	Resistant to race 0
Scotland	Universal susceptible	Craig's Royal	No	No
	R1	Craig's Snow White	Yes	Yes
	R2	1512 c(16)	No	Yes
	R3	Pentland Ace	No	Yes
	R4	1563 c(14)	No	(Yes)[a]
	R5	3053–18	**Yes**[b]	Yes
	R6	XD2–21	**Yes**	Yes
	R7	2182ei(7)	No	Yes
	R8	2424a(5)	No	Yes
	R9	2573(2)	**Yes**	Yes
	R10	3681ad(1)	No	Yes
	R11	5008ab(6)	No	Yes
	R1R2	1647b(1)	Yes	Yes
	R1R3	2070(32)	Yes	Yes
	R1R4	1506b(9)	Yes	Yes
	R2R3	1682 c(1)	No	Yes
	R2R4	2070(31)	No	Yes
	R3R4	1488b(1)	No	Yes
	R1R2R3	2070(59)	Yes	Yes
	R1R2R4	2070(69)	Yes	Yes
	R1R3R4	2070(4)	Yes	Yes
	R2R3R4	2070(50)	No	Yes
	R1R2R3R4	2070(54)	Yes	Yes
Netherlands	R1	CEBECO-43154-5, CIP800986	Yes	Yes
	R2	CEBECO-44158-4, CIP800987	No	Yes
	R3	CEBECO-4642-1, CIP800988	No	Yes
	R4	CEBECO-4431-5, CIP800989	No	(Yes)[a]
	R8	Black 2424a(5), CIP800993	No	Yes
	R9	Black 2573, CIP800994	**Yes**	Yes
	R10	Black 3618ad(1), CIP800995	No	Yes
	R11	Black 5008ab(6), CIP800996	No	Yes
	R1R2	CIP800997	Yes	Yes
	R1R3	CIP800998	Yes	Yes
	R2R3	CIP801000	No	Yes
	R3R4	CIP801002	No	Yes
	R1R2R3	CIP801003	Yes	Yes
	R2R3R4	CIP801005	No	Yes
		Arletta (1998)[c]	No	n.d.
		Kuras (1996)	No	n.d.
Austria		Evita (1995)	No	No
		Gabriella (2000)	No	n.d.
		Linzer Delikatess (1975)	Yes	No
		Romina (1989)	No	n.d.
		Rosita (1999)	No	n.d.
Germany		Laura (1998)	Yes	n.d.
		Quarta (1979)	No	n.d.

[a]The race 0 isolate produced small, slowly sporulating lesions on the R4 differentials from different sources indicating that it may possess incomplete avirulence for this resistance factor.
[b]Unexpected phenotype indicated in bold-face. n.d.; not done.
[c]Year of release indicated in parentheses.

R1 is absent in this cultivar. The German cultivar Laura (launched in 1998) also has the *R1* allele; however, its phenotype of resistance to race 0 was not tested. According to varietal assessment results (Anonymous, 2001), the level of susceptibility to foliage blight of Laura (*R1* carrier, second-early maturity) in the field is low–intermediate. For comparison, this level of susceptibility corresponds to a score of 4 on the German 1–9 scale of blight susceptibility (Anonymous, 2003). Laura proved more field-resistant than the other second-early, non-*R1*-carrying cultivars

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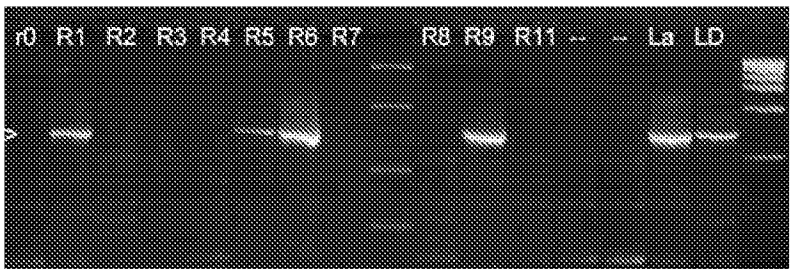


Figure 1 PCR-amplified *R1* fragment (1400 bp, marked at left) from genomic DNA of potato R-gene differentials and cvs Laura and Linzer Delikatess using *R1*-specific primers. r0, universal susceptible differential (Craig's Royal); R1–R11, Scottish R-gene differentials; La, cv. Laura; LD, cv. Linzer Delikatess. Size standards: centre, Fermentas FastRuler; at right, Invitrogen high DNA mass ladder.

Table 2 Summary of *Phytophthora infestans* avirulence phenotypes on R-gene differentials from published studies on isolates collected between 1983 and 1998

Source	Total isolates	Avirulent on R1	Avirulent on R1 and virulent on R5, R6, R9	Country	Year of collection	Host
Hermansen *et al.*, 2000	374	24	0	Finland, Norway	1990–96	potato
Andrivon *et al.*, 1994	23	1	0	France	1991	potato
Lebreton & Andrivon, 1998	62	1	0	France	1995/96	potato, tomato
Drenth *et al.*, 1994	253	not indicated		Netherlands	1981–91	
Schöber & Turkensteen, 1992	77	12	0	Netherlands	1988	potato
Flier & Turkensteen, 1999	36	7	0	Netherlands	1995	potato
Sujkowski *et al.*, 1996	95	2	0	Poland	1985–91	potato
Elansky *et al.*, 2001	191	5	not indicated	Russia	1997/98	potato, tomato
Abu-El Samen *et al.*, 2003[a]	21	9	1 (on R6)	USA	1994/95	potato
Deahl *et al.*, 1991	10	1	0	USA, Canada	1983–89	potato, tomato
Dorrance *et al.*, 1999	60	4	3 (1 on R5, 1 on R6, 1 on R5+R6)	USA	1996/97	potato, tomato, nightshades
Goodwin *et al.*, 1995	32	6	1 (R5), (R6 not done)	USA, Canada, Mexico	1989–94	potato, tomato
Rivera-Peña, 1990	78	23	9 (5 on R5, R6 not done, 4 on R9)	Mexico	1985/86	wild potatoes
Forbes *et al.*, 1997	108	18	1 (R5)	Ecuador	1993	potato
Oyarzun *et al.*, 1998	60	0	0	Ecuador	1993–95	potato
Oyarzun *et al.*, 1998	60	57	0	Ecuador	1994–96	tomato
Total[b]	1287	170	15			

[a]These authors investigated single-zoospore isolates from two single-lesion isolates; all other authors had single-lesion isolates.
[b]Excluding Drenth *et al.*, 1994.

investigated, Evita and Quarta, which possess an intermediate susceptibility level (score 5). Romina (early) also is intermediately susceptible, whereas the late-maturing cv. Kuras is resistant in the field. Both early-maturing cultivars, Rosita and the silent *R1* carrier Linzer Delikatess display an intermediate-pronounced level of susceptibility in the field (score 6). The first-early cultivar Gabriella has been scored intermediately susceptible and no results are available for Arletta from the same source (Anonymous, 2001).

The experiment reported here confirms that the *R1* gene is distributed across all differentials specific for the R1 factor and for all combinations of R1 with other factors. Therefore, there is a good chance that the R1 phenotype of resistance is expressed by the functional *R1* allele as isolated by Ballvora *et al.* (2002). Gebhardt *et al.* (2004) detected this allele also by its distinctive 1400 bp PCR fragment in several accessions of the Mesoamerican *S. demissum* and in one accession of *S. stoloniferum*.

The occurrence of the *R1* allele in the R5, R6 and R9 differentials was unexpected (Table 1, Fig. 1). Because the phenotype resulting from interaction with the universally avirulent race 0 does not allow discrimination among different *R* genes, it cannot be assessed whether and to what extent *R1* may contribute to the resistance phenotype displayed by these three R-gene differential clones.

In Table 2, results of 15 studies on avirulence of *P. infestans* isolates collected throughout more than one decade are summarized. Only 170 out of 1287 isolates tested (13%) were avirulent on the R1 differentials and only 15 of these 170 isolates (9%) were virulent on R5, R6, or R9 differential clones. The latter were exclusively reported in studies from the Americas where R-gene differential clones different from those analysed in this study may have been partly used (as an example see Goodwin *et al.*, 1995). However, as the status of *P. infestans* avirulence generally has been assessed by the resistance phenotypes of R-gene differential clones, it is possible that those

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multiply-virulent pathotypes may, in part, be artefacts for which inadvertent selection has occurred. This could be verified once additional R-gene differentials of R5, R6, and R9 devoid of *R1* become available. Such additional differentials could easily be obtained by selecting self- and cross-progenies of the existing differentials against R1 using the molecular markers now available (Gebhardt *et al.*, 2004).

Two out of nine commercial potato cultivars tested positive for the presence of *R1* (Table 1) indicating that *R1* still occurs within contemporary European potato breeding stocks although races breaking it down had already appeared in Europe by 1932 (Lehmann, 1937). Selection for this gene was suspended half a century ago when the widespread breakdown of the resistance to several of these *R* genes was recognized (Ross, 1986). The presence of *R1*, however, is understandable considering the narrow pedigree-breeding schemes widely applied, the long period of generation turnover in potato breeding (Gebhardt *et al.*, 2004), and no directed selection against *S. demissum* *R* genes. Notwithstanding, there is evidence that defeated *R* genes still may contribute to an overall increased level of resistance in the field (Darsow *et al.*, 1987; Stewart *et al.*, 2003). Therefore, breeders could be encouraged to use *R* genes for resistance to late blight particularly when these are easily selected as in the case of the *R1* allele.

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14/112,747	12/23/2013	Willem Hendrik Lindhout	091876-0027	5919

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Washington, DC 20001

EXAMINER
BOGGS, RUSSELL T

ART UNIT	PAPER NUMBER
1663	

NOTIFICATION DATE	DELIVERY MODE
03/14/2016	ELECTRONIC

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

mweipdocket@mwe.com

Office Action Summary	Application No. 14/112,747	Applicant(s) LINDHOUT ET AL.	
	Examiner RUSSELL BOGGS	Art Unit 1663	AIA (First Inventor to File) Status No

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE *3* MONTHS FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☒ Responsive to communication(s) filed on *18 October 2013*.

☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on _____.

2a)☐ This action is **FINAL**. 2b)☒ This action is non-final.

3)☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on _____; the restriction requirement and election have been incorporated into this action.

4)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims*

5)☒ Claim(s) *1-18* is/are pending in the application.

5a) Of the above claim(s) _____ is/are withdrawn from consideration.

6)☐ Claim(s) _____ is/are allowed.

7)☒ Claim(s) *1-18* is/are rejected.

8)☐ Claim(s) _____ is/are objected to.

9)☐ Claim(s) _____ are subject to restriction and/or election requirement.

* If any claims have been determined allowable, you may be eligible to benefit from the **Patent Prosecution Highway** program at a participating intellectual property office for the corresponding application. For more information, please see http://www.uspto.gov/patents/init_events/pph/index.jsp or send an inquiry to PPHfeedback@uspto.gov.

Application Papers

10)☒ The specification is objected to by the Examiner.

11)☒ The drawing(s) filed on *18 October 2013* is/are: a)☒ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

Priority under 35 U.S.C. § 119

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

Certified copies:

a)☐ All b)☐ Some** c)☐ None of the:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

** See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☒ Notice of References Cited (PTO-892)

2) ☒ Information Disclosure Statement(s) (PTO/SB/08a and/or PTO/SB/08b) Paper No(s)/Mail Date _____.

3) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date. _____ .

4) ☐ Other: _____.

DETAILED ACTION

The present application is being examined under the pre-AIA first-to-invent provisions.

Status

1. Claims 1-18, filed 18 October 2013, are examined herein.

Examiner's Note

2. The Examiner notes the amended version of Table 5A with sequence identifiers.

Information Disclosure Statement

3. The listing of references in the specification at pages 93-101 is not a proper information disclosure statement. 37 CFR 1.98(b) requires a list of all patents, publications, or other information submitted for consideration by the Office, and MPEP § 609.04(a) states, "the list may not be incorporated into the specification but must be submitted in a separate paper." Therefore, unless the references have been cited by the examiner on form PTO-892 or -1449, they have not been considered.

Specification

4. The disclosure is objected to because of the following informalities.

The disclosure is objected to because it contains an embedded hyperlink and/or other form of browser-executable code. Applicant is required to delete the embedded

hyperlink and/or other form of browser-executable code. See MPEP § 608.01. See page 9, line 17; page 53, line 5 and line 19; and page 95, line 3.

The specification is objected to because it recites the question "VAN WELKE ACESSIE ????" with no context in the middle of page 75.

The specification lists non-patent literature in Table 10 on pages 84-90. Several of the claim terms in claims 1 and 5 are rejected under 35 USC 112(b) as being indefinite because they lack an art-accepted meaning. Applicant apparently intended to define the claim terms by reference to this table. It is the Office's interpretation that it is Applicant's clear intent to incorporate the essential material cited by reference to the non-patent literature into the claims. The meaning and scope of the claim limitations can only be understood by reference to listed references, however, such an incorporation is improper.

The incorporation of essential material into specification by reference to an unpublished U.S. application, foreign application or patent, to a publication, or to an online database is improper. Applicant is required to amend the specification to include the material incorporated by reference, if the material is relied upon to overcome any objection, rejection, or other requirement imposed by the Office. The amendment must be accompanied by a statement executed by the applicant, or a practitioner representing the applicant, stating that the material being inserted is the material

previously incorporated by reference and that the amendment contains no new matter. 37 CFR 1.57(f).

The specification is objected to because it lacks the address of NCIMB on pages 34-35 where the specification provides information regarding the deposits.

Appropriate correction is requested.

Claim Objections

5. Claims 1-3 and 5-6 are objected to because of the following informalities.

Claims 1 and 5 are objected to for several reasons.

First, "Solanum spegazzinii" and "Solanum tarijense" in line 9 of claim 1 and line 7 of claim 5(f) are not italicized.

Second, the word "and" in line 13 of claim 1 and line 11 of claim 5(f) is grammatically inappropriate because it is not at the end of the Markush group – if it was intended to apply to a sub-Markush group, the sub-grouping is not clear. Applicant doesn't, for example, use semi-colons within the Markush groups of claim 1 and claim 5 to distinguish groupings within the claim.

Third, they recite the limitation "the expressed sequence tags EST724115 and EST717227 for asparagine synthetase" in line 18 of claim 1 and line 16 of claim 5(f). The Examiner believes that Applicant intended to reference the gene defined by those

two ESTs, but as worded, the limitation only requires the two ESTs themselves. One of skill in the art would believe that a plant that was transgenic for just the two ESTs would not show a change in phenotype.

Claims 2-3 are objected to because they recite the subject composition in the plural rather than reading on a single composition. In other words, claim 2 reads on plural “seed” and claim 3 reads on plural "plants."

Claim 6 is objected to because the words “accession number” are missing between “NCIMB” and each of “41663,” “41664,” “41665,” and “41765” in line 2.

Appropriate correction is requested.

Claim Rejections - 35 USC § 112

The following is a quotation of 35 U.S.C. 112(b):

> (B) CONCLUSION.—The specification shall conclude with one or more claims particularly pointing out and distinctly claiming the subject matter which the inventor or a joint inventor regards as the invention.

The following is a quotation of 35 U.S.C. 112 (pre-AIA), second paragraph:

> The specification shall conclude with one or more claims particularly pointing out and distinctly claiming the subject matter which the applicant regards as his invention.

6. Claims 1-18 are rejected under 35 U.S.C. 112(b) or 35 U.S.C. 112 (pre-AIA), second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which the inventor or a joint inventor, or for pre-AIA the applicant regards as the invention.

Claims 1 and 5 are rejected because they recite numerous limitations, for example "*S. demissum* R2 and R2-like protein genes," in which genes are identified by a variety of terms where the meaning of the terms are indefinite. Terms such as "R2" and "R2-like" lack a general art-accepted meaning and Applicant does not explicitly define the terms in the specification. For example, there are two different "R2"s in the list below. Applicant provides Table 10 on pages 84 to 90 which cite to references from the literature, however, the incorporation of essential material in the specification by reference to an unpublished U.S. application, foreign application or patent, or to a publication is improper. 37 C.F.R. 1.57(c).

The terms in question include:

resistance to *Phytophthora infestans* from *Solarium bulbocastanum or S. demissum*,

resistance to *G. rostochiensis* Ro1 from *S. tuberosum* subsp. *andigena* CPC 1690 or 1673,

resistance to *G. pallida* from *S. tuberosum* subsp. *andigena*,

resistance to *G. rostochiensis* Ro5-22 and *G. pallida* Pa2-D383 from *S. tuberosum* accession 78-3778,

resistance to *G. pallida* Pa2 and Pa3 from *Solanum spegazzinii* and *Solanum tarijense*,

resistance to *G. pallida* Pa3 from *S. tuberosum* accession 3704-76,

resistance to *M. chitwoodi* from *S. bulbocastanum*,

resistance to *M. chitwoodi* / *M. fallax* from *S. fendlerii* and *S. hougassii*,

resistance to *M. hapla* from *S. chacoense* and *S. tarijense*,

resistance to PVX from *S. tuberosum* cv Pentland Ivory,

resistance to PVS from *S. tuberosum* DW 83-3121,

beta-carotene hydroxylase 2 (CHY2) gene;

zeaxanthin epoxidase (ZEP) gene;

potato gene for granule-bound starch synthase;

gene for beta-fructofuranosidase or potato acid invertase;

sucrose-phosphate-synthase gene;

gene for the putative invertase inhibitor precursor (INH1/INH2);

gene for tuber polyphenol oxidase PPO;

sucrose synthase gene;

chlorogenate inducer gene;

expressed sequence tags EST724115 and EST717227 for asparagine synthetase;

phosphorylase-L gene;

gene for R1 protein or glucan water dikinase, all from *S. tuberosum*;

S. demissum R2 and R2-like protein genes;

S. hjertingii R2 late blight resistance protein (Rpi-hjt1.3) gene;

ABPT Rpi protein gene;

S. venturii late blight resistance protein genes;

S. bulbocastanum putative disease resistant protein RGA2 gene;

S. bulbocastanum blight resistance protein RPI gene;

S. bulbocastanum late blight resistance protein Rpi-b1b2 (Rpi-blb2) gene;

S. bulbocastanum NBS-LRR resistance protein (Rpi-bt1) gene;

S. tuberosum nematode resistance protein (Gro1-4) gene;

S. tuberosum disease resistance protein Gpa2 gene;

S. tuberosum subsp. *andigena* ADG212x(v-2)7a gene controlling resistance to potato Y and A potyviruses;

S. tuberosum subsp. *andigena* ry-1 gene for resistance gene-like, exons 1-6, splice variants C38 and C19;

dihydroflavonol 4-reductase gene;

flavonoid 3′,5′-hydroxylase gene;

gene for the R2R3 MYB transcriptional regulator of anthocyanin-1 production pathway

all from *S. tuberosum*

S. demissum late blight resistance protein (R1) gene;

potato late blight resistance protein R3a gene from *S. tuberosum L.*; and

S. bulbocastanum NBS-LRR resistance protein (Rpi-bt1) gene.

Because of the indefiniteness of these terms , the metes and bounds of the claims cannot be determined.

Furthermore, the meaning of these terms could arbitrarily change to designate something different during the lifetime of a patent. Thus, one's ability to determine the metes and bounds of the claim would be impaired. See *In re Hammack*, 427 F .2d 1378, 1382; 166 USPQ 204, 208 (CCPA 1970).

Claims 1 and 5 are also rejected because several terms lack antecedent basis. Claim 1 recites the following limitations between line 13 and the last line and claim 5(f)

recites the limitations between line 11 and the last line:

the beta-carotene hydroxylase 2 (CHY2) gene;

the zeaxanthin epoxidase (ZEP) gene;

the potato gene for granule-bound starch synthase;

the gene for beta-fructofuranosidase or potato acid invertase;

the sucrose-phosphate-synthase gene;

a gene for the putative invertase inhibitor precursor (INH1/INH2);

the gene for tuber polyphenol oxidase PPO;

the sucrose synthase gene;

the chlorogenate inducer gene;

the expressed sequence tags EST724115 and EST717227 for asparagine synthetase;

the phosphorylase-L gene;

the gene for R1 protein or glucan water dikinase, all from *S. tuberosum*;

the *S. demissum* R2 and R2-like protein genes;

the *S. hjertingii* R2 late blight resistance protein (Rpi-hjt1.3) gene;

the ABPT Rpi protein gene;

the *S. venturii* late blight resistance protein genes;

the *S. bulbocastanum* putative disease resistant protein RGA2 gene;

the *S. bulbocastanum* blight resistance protein RPI gene;

the *S. bulbocastanum* late blight resistance protein Rpi-b1b2 (Rpi-blb2) gene;

the *S. bulbocastanum* NBS-LRR resistance protein (Rpi-bt1) gene;

the *S. tuberosum* nematode resistance protein (Gro1-4) gene;

the *S. tuberosum* disease resistance protein Gpa2 gene;

the *S. tuberosum* subsp. *andigena* ADG212x(v-2)7a gene controlling resistance to potato Y and A potyviruses;

and/or the *S. tuberosum* subsp. *andigena* ry-1 gene for resistance gene-like, exons 1-6, splice variants C38 and C19;

the dihydroflavonol 4-reductase gene;

the flavonoid 3′,5′-hydroxylase gene;

and the gene for the R2R3 MYB transcriptional regulator of anthocyanin-1 production pathway all from *S. tuberosum*

the *S. demissum* late blight resistance protein (R1) gene;

the potato late blight resistance protein R3a gene from *S. tuberosum L.*; and

and the *S. bulbocastanum* NBS-LRR resistance protein (Rpi-bt1) gene.

None of the above limitations have an antecedent basis, and the gene to which the limitations refer is indefinite. For a specific example, claim 5(f) line 15 recites sucrose synthase without specifying a source and thus reads on the universe of all genes that arguably have sucrose synthase activity. Certain of the limitations recite a species source, e.g. “the gene for R1 protein or glucan water dikinase, all from *S. tuberosum*,” but this hardly identifies the gene being used. As taught by Applicant, “the tetraploid genome [of a potato] is extremely heterozygous, often harbouring multiple alleles per locus.” Specification, p. 2, ll. 27-28. Additionally, a recent review by Spooner et al. teaches that "[h]undreds of successful interspecific hybrids have been reported in the

literature" and that "the biological concept of a species is difficult to apply to potatoes." Spooner et al., Bot Rev 80:283-383 (2014), p. 303 & see also pp. 303-18.

Claims 1 and 5 are further rejected because they each recite the limitation "the *S. tuberosum* subsp. *andigena* ADG212x(v-2)7a gene controlling resistance to potato and A potyviruses; and/or the *S. tuberosum* subsp. *andigena* ry-1 gene for resistance gene-like, exons 1-6, splice variants C38 and C19" in lines 25-28 of claim 1 and lines 24-26 of claim 5(f). How the recited "and/or" is intended to be construed is indefinite – does it apply only to "the *S. tuberosum* subsp. *andigena* ADG212x(v-2)7a gene controlling resistance to potato and A potyviruses" and "*S. tuberosum* subsp. *andigena* ry-1 gene for resistance gene-like, exons 1-6, splice variants C38 and C19" or does it include other members of the Markush group?

Furthermore, the meaning of "*S. tuberosum* subsp. *andigena* ry-1 gene for resistance gene-like, exons 1-6, splice variants C38 and C19" is also indefinite.

Claim 4 recites the limitation "the plant" in line 1. There is insufficient antecedent basis for this limitation in the claim because the subject of claim 1 is a potato line comprising multiple plants. So which plant is being referenced is indefinite.

A broad range or limitation together with a narrow range or limitation that falls within the broad range or limitation (in the same claim) is considered indefinite, since the resulting claim does not clearly set forth the metes and bounds of the patent

protection desired. See MPEP § 2173.05(c). Note the explanation given by the Board of Patent Appeals and Interferences in *Ex parte Wu*, 10 USPQ2d 2031, 2033 (Bd. Pat. App. & Inter. 1989), as to where broad language is followed by "such as" and then narrow language. The Board stated that this can render a claim indefinite by raising a question or doubt as to whether the feature introduced by such language is (a) merely exemplary of the remainder of the claim, and therefore not required, or (b) a required feature of the claims. Note also, for example, the decisions of *Ex parte Steigewald*, 131 USPQ 74 (Bd. App. 1961); *Ex parte Hall*, 83 USPQ 38 (Bd. App. 1948); and *Ex parte Hasche*, 86 USPQ 481 (Bd. App. 1949). In the present instance, claim 7 recites the broad recitation “diploid potato breeding line” in line 1, and the claim also recites “more preferably an elite line” also in line 1 which is the narrower statement of the range/limitation.

Claim 7 is also rejected as indefinite because it recites the limitation “obtainable“ in lines 1-2 in relation to the method of claim 5. It is indefinite how to identify a future result of the claimed method and thus the metes and bounds of the claim cannot be determined. This aspect of the rejection would be obviated by amending “obtainable" to “obtained.”

Claim 8 recites the limitation "said parent plants" in lines 1-2. There is insufficient antecedent basis for this limitation in the claim because there is no prior reference to parent plants. So which plant is being referenced is indefinite. An amendment

changing “one of said parent plants” to “either said first or second potato plant“ would obviate this aspect of the rejection.

Dependent claims are included in this rejection because none provide limitations obviating this rejection.

The following is a quotation of 35 U.S.C. 112(a):

> (a) IN GENERAL.—The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same, and shall set forth the best mode contemplated by the inventor or joint inventor of carrying out the invention.

The following is a quotation of 35 U.S.C. 112 (pre-AIA), first paragraph:

> The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same and shall set forth the best mode contemplated by the inventor of carrying out his invention.

7. Claims 1-6 and 7-18 are rejected under 35 U.S.C. 112(a) or 35 U.S.C. 112 (pre-AIA), first paragraph, as failing to comply with the written description requirement. The claim(s) contains subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor or a joint inventor, or for pre-AIA the inventor(s), at the time the application was filed, had possession of the claimed invention.

Claim 1 is drawn to a diploid, fertile, self-compatible and essentially homozygous potato line having an average tuber yield expressed in grams of fresh weight of at least 200 grams per plant; wherein said line additionally comprises 1-6 agronomically desirable traits or genes selected from a Markush group. Claims depending from claim

1 read on hybrid potato seed produced from a cross between two essentially homozygous parental breeding lines and potato plants produced therefrom. Claim 8 reads on a method using a plant from claim 1. Claims 9-13 read on plants and plant parts produced by the method of claim 8. Claim 14 reads on a tissue culture produced by the plant of claim 10 and claim 15 reads on a plant produced from the tissue culture of claim 14. Claim 16 reads on a method using a seed produced according to claim 8 to produce a plant; claim 17 reads on a plant or tuber produced according to claim 16. Claim 18 reads on a set of at least 2 intercrossable diploid, fertile, self-compatible and essentially homozygous potato lines comprising plants according to claim 1.

Claim 5 is a method claim drawn to producing a diploid, fertile, self-compatible and essentially homozygous potato line starting with an uncharacterized first potato plant of a first diploid self-compatible, fertile and essentially homozygous potato line and crossing it to a second potato plant where the second potato plant has an average tuber yield expressed in grams of fresh weight of at least 200 grams per plant and then selfing or backcrossing to provide a diploid, self-compatible and essentially homozygous potato breeding line comprising plants having an average tuber yield of at least 200 grams per plant, and then introducing into a plant of said line at least one trait or gene selected from a Markush group. Claim 6 recites specific deposited cell lines; claim 7 encompasses a diploid potato breeding line obtainable by the method of claim 5.

In the specification, Applicant describes obtaining the IVP07-1001/4 line from publically available germplasm and plants that contain the *Sli* gene and using these plants to breed AGVD1, AGVD2, and AGVD3. Specification ("Spec."), pp. 46-47.

Applicant describes the deposited lines by breeders reference: AGVD1 (NCIMB 41663), AGVD2 (NCIMB 41664), AGVD3 (NCIMB 41665) and AGVD17 (NCIMB 41765). *Id.*, pp. 34-35. Applicant describes a breeding process according to the instant invention starting with AGVD1-3 or AGVD17 on page 39 *et seq.*

Applicant describes crossing the *Sli* gene donor with various potato clones designated: IVP97-079-9, IVPAA-096-I8 and SH83-92-488 to generate clones D1, D2 and D3. *Id.*, p. 49. Applicant describes creating a line containing the Sli gene with enhanced agricultural quality in Example 2, pp. 48-51. Applicant describes creating a molecular marker platform, i.e., SNPs, in Example 3, p. 51 *et seq.* In Example 4, Applicant describes the development of homozygous diploid potato plants and then F1 hybrid progeny, as well as subsequent generations. *Id.*, p. 60 *et seq.* Applicant describes the meaning of the claim term "essentially homozygous" on pages 21-22 of the instant specification and suggests that the term reflects a level of homozygosity that is above 25% or 75%. *Id.*, p. 21-22. Applicant generally describes adding an agronomically desirable trait to a potato genotype on, e.g. page 37.

To claim a genus under the written description requirement, Applicant is required to describe a representative number of species to reflect the variation within the genus or structures sufficient to define the genus. The factors to be considered include disclosure of complete or partial structure, physical and/or chemical properties, functional characteristics, structure/function correlation, methods of making the claimed product, or any combination thereof.

The claimed genera, in particular, include those set forth in the following exemplary claims:

claim 1 – any “diploid, fertile, self-compatible and essentially homozygous potato line” with a certain recited characteristic together with at least one agronomically desirable trait;

claim 5 – any “diploid, fertile, self-compatible and essentially homozygous potato line” as a starting material in part (a) to produce a “diploid, fertile, self-compatible and essentially homozygous potato line” with an added trait or gene.

Applicant fails to describe a representative number of species in comparison to the size of the genus claimed. In the instant application, Applicant describes only four potato lines to use in the claimed methods or as a starting point to obtain the claimed compositions, and all were created from the same line, the IVP07-1001/4 line. Applicant fails to describe working examples of the broad genus of other potato lines falling within the scope of the claims. Further, Applicant only describes one parental line and fails to describe examples of claimed potato lines descended from different parents.

However, in contrast to the limited description, as noted above, Applicant claims a large genus of potato plants. Jansky and Spooner et al. describes the breadth of the of the claimed genus as extremely large. See, e.g., Jansky, "Breeding, Genetics, and Cultivar Development," Advances in Potato Chemistry and Technology, ed. J. Singh & L. Kaur, pp. 27-62 (June, 2009), pp. 27-29; Spooner et al., Bot Rev 80:283-383 (2014),

Table 1, pp. 286-94. Applicant's claimed invention reads on any potential potato line that is a "diploid, fertile, self-compatible and essentially homozygous potato line."

Applicant fails to describe a representative number of species in comparison to the size of the genus claimed. Applicant also fails to describe any structure necessary and/or sufficient for a potato line to be used in the claims. The specification therefore fails to provide an adequate written description to support the breadth of the claims. Applicant has not demonstrated possession to one of skill in the art of the instant invention as broadly as claimed at the time of filing, and one of skill in the art would not believe Applicant to be in possession of the genus as broadly as claimed.

Dependent claims are included in this rejection because none provide further limitations obviating this rejection.

8. Claims 1-18 are also rejected under the written description requirement because claims 1 and 5 make reference to database accession numbers in the claims: EST724115 and EST717227, and because several of the claim terms are defined only in reference to Table 10 on pages 84-90. It is the Office's interpretation that it is Applicant's clear intent to incorporate the essential material cited by reference to the accession numbers and Table 10 into the claims. The meaning and scope of the claim limitations can only be understood by reference to the accession numbers and Table 10, however, such an incorporation is improper.

The incorporation of essential material in the claims by reference to an unpublished U.S. application, foreign application or patent, to a publication, or to an online database is improper. Applicant is required to amend the claims to include the material incorporated by reference, if the material is relied upon to overcome any objection, rejection, or other requirement imposed by the Office. The amendment must be accompanied by a statement executed by the applicant, or a practitioner representing the applicant, stating that the material being inserted is the material previously incorporated by reference and that the amendment contains no new matter. 37 CFR 1.57(f).

Dependent claims are included in this rejection because none provide further limitations obviating this rejection.

9. Claims 1-18 are rejected under 35 U.S.C. 112(a) or 35 U.S.C. 112 (pre-AIA), first paragraph, as failing to comply with the enablement requirement. The claim(s) contains subject matter which was not described in the specification in such a way as to enable one skilled in the art to which it pertains, or with which it is most nearly connected, to make and/or use the invention.

The invention appears to employ novel plants. Since the plant is essential to the claimed invention it must be obtainable by a repeatable method set forth in the specification or otherwise be readily available to the public. If the plant is not so obtainable or available, the requirements of 35 USC § 112 may be satisfied by a deposit

of the seeds. A deposit of 2500 seeds of each of the claimed embodiments is considered sufficient to ensure public availability.

The specification does not disclose a repeatable process to obtain the plant and thus it is not apparent if the plant is readily available to the public.

(a) If a deposit is made under the terms of the Budapest Treaty, then a statement, affidavit or declaration by Applicant, or a statement by an attorney of record over his or her signature and registration number, or someone empowered to make such a statement, stating that the instant invention will be irrevocably and without restriction released to the public upon the issuance of a patent, would satisfy the deposit requirement made herein.

(b) If a deposit has not been made under the Budapest Treaty, then in order to certify that the deposit meets the criteria set forth in 37 CFR 1.801-1.809 and MPEP 2402-2411.05, Applicant may provide assurance of compliance by statement, affidavit or declaration, or by someone empowered to make the same, or by a statement by an attorney of record over his or her signature and registration number showing that:

(i) during the pendency of this application, access to the invention will be afforded to the Commissioner upon request;

(ii) all restrictions upon availability to the public will be irrevocably removed upon granting of the patent in accordance with 37 CFR § 1.808(a)(2);

(iii) the deposit will be maintained in a public depository for a period of 30 years or 5 years after the last request or for the effective life of the patent, whichever is longer;

(iv) a test of the viability of the biological material at the time of deposit (see 37 CFR

§ 1.807); and,

(v) the deposit will be replaced if it should ever become inviable.

In addition, the identifying information set forth in 37 CPR 1.809(d) should be added to the specification, if not already present. See 37 CPR 1.801 - 1.809 (MPEP 2401-2411.05) for additional explanation of these requirements.

Applicant has deposited seeds (see e.g. pages 34-35 of the instant specification). There is no evidence in the record that that the requisite number of seeds, 2,500, was deposited. Examiner also notes that, *inter alia*, a statement, affidavit or declaration by Applicant, or a statement by an attorney of record over his or her signature and registration number, or someone empowered to make such a statement, stating that the instant invention will be irrevocably and without restriction released to the public upon the issuance of a patent is required. If the deposit was not made under the Budapest Treaty all of (b)(i)-(v) must be affirmed.

Claim Rejections - 35 USC § 103

The following is a quotation of 35 U.S.C. 103(a) which forms the basis for all obviousness rejections set forth in this Office action:

> (a) A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102 of this title, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains. Patentability shall not be negatived by the manner in which the invention was made.

10. Claims 1-5 and 7-18 are rejected under pre-AIA 35 U.S.C. 103(a) as being unpatentable over Jansky, "Breeding, Genetics, and Cultivar Development," Advances

in Potato Chemistry and Technology, ed. J. Singh & L. Kaur, pp. 27-62 (June, 2009) in view of Phumichai et al., Genome 48:977-84 (2005), in further view of Maris, Euphytica 46:15-33 (1990), Simmonds, Potato Res 40:191-214 (1997), and view of Watanabe et al., Breed Sci 46:329-36 (1996) and in further view of Bradshaw, New and Modern Methods of Potato Breeding, pp. 157-77 (2007).

The factual inquiries set forth in *Graham v. John Deere Co.*, 383 U.S. 1, 148 USPQ 459 (1966), that are applied for establishing a background for determining obviousness under 35 U.S.C. 103(a) are summarized as follows:

a. Determining the scope and contents of the prior art.

b. Ascertaining the differences between the prior art and the claims at issue.

c. Resolving the level of ordinary skill in the pertinent art.

d. Considering objective evidence present in the application indicating obviousness or nonobviousness.

Jansky teaches the breeding strategy of first obtaining homozygous diploids using a self-incompatibility inhibitor. Jansky, p. 46. Applicant has obtained homozygous diploids using the *Sli* gene self-incompatibility inhibitor. E.g., spec., pp. 34, 39. Jansky teaches that "[h]ighly homozygous inbreds could be also be used for heterosis breeding. Jansky, p. 46. Jansky teaches that such an approach might be difficult, but does not teach that it would be impossible. *Id.*

Additionally, Simmonds teaches that the production of potatoes by seed is a valuable technique because such potatoes are disease-free, and thus provides motivation (p. 194, p. 202); production of potatoes from seed is routinely used in the

Third World. Simmonds, pp. 202-03. Simmonds also teaches that diploid potatoes were underutilized at the time of Simmonds' review and thus further provides motivation to develop diploid potatoes. *Id.*, e.g., p. 192, p. 209. Simmonds teaches that heterosis produces a "spectacular" increase in yield. p. 192.

None of the above references teach a diploid, fertile, self-compatible and essentially homozygous potato line having an average tuber yield expressed in grams of fresh weight of at least 200 grams per plant. However, it would have been *prima facie* obvious to one of ordinary skill in the art at the time of Applicants' invention to follow the teachings of Jansky and Simmonds to develop a diploid, fertile, and essentially homozygous potato line. Developing the line from, e.g. the species *Solanum tuberosum* or limitation "*Solanum tuberosum* comprising introgression segments of other tuber-bearing *Solanum* species crossable to *Solanum tuberosum,*" would be obvious in view of, e.g., the importance of this species in world agriculture. See, e.g., Spec., p. 1, ll. 13-15. Given the level of skill in the art at the time of Applicants' invention, one of ordinary skill in the art would have had a reasonable expectation of success.

Additionally, Phumichai et al. confirms the approach of Jansky, and documents the use of the *Sli* gene in the approach. Phumichai et al., abstract.

> Potato (Solanum tuberosum) is an auto- [sic] or segmental allo-tetraploid showing tetrasomic inheritance, which results in complicated genetic segregation. Since potatoes are propagated vegetatively, highly heterotic genotypes can be maintained, which facilitates the use of heterosis, an essential factor in potato breeding, particularly for tuber yield. Alternatively, open-pollinated true potato seed (TPS) can be used as a low-cost planting material for potato production.

> Highly homozygous inbreds, once amply produced, would be highly useful for the development of TPS varieties with genetic uniformity, as well as for the investigation of heterosis."
>
> "A scheme for hybrid breeding using homozygous diploids and subsequent somatic hybridization has been proposed, for which the availability of highly homozygous diploid (or pure) lines is a key for success. In considering the use of pure lines for the development of genetically uniform [true potato seed] varieties, self-fertile, seed-propagated lines are highly desirable, and simple techniques to develop such lines are necessary." .

Id., pp. 977-78 (citations omitted). Therefore, introducing, e.g., the *Sli* gene as taught by Phumichai et al. into an self-incompatible cultivated diploid potato line so as to allow self-compatibility would have been obvious one of skill in the art at the time of Applicant's invention. See, e.g., *Id.* Phumichai et al. teaches using lines with a 50% homozygosity (see, e.g., page 979), which would be further consistent with an inbred line.

Claim 1 recites further limitations directed at properties of the claimed plants such as tuber yield. Selecting potato plants for increased tuber yield is obvious. Furthermore, Jansky teaches that properties of potato plants are environmentally variable for traits including yield and tuber size. Jansky, p. 45. In an example, Maris teaches 2x plants that have an average tuber yield of at least 200 g/plant. See, e.g., Maris, Table 8, pp. 26-28. Finally, achieving a certain degree of homozygosity, e.g. 50%, in a plant line is implicit in inbreeding. See, e.g., Jansky, p. 46.

Additionally, where the claimed and prior art products are identical or substantially identical in structure or composition, or are produced by identical or substantially identical processes, a *prima facie* case of either anticipation or

obviousness has been established. *In re Best*, 562 F.2d 1252, 1255, 195 USPQ 430, 433 (CCPA 1977). The burden of proof is then on the applicant to demonstrate that the product does not necessarily possess the properties of the claimed invention. *Id.*, pp. 195 USPQ 433-34. See, also, MPEP section § 2112 (v). Further, in 2009, the Federal Circuit returned to this question – does a previously undiscovered property of a composition that was otherwise obvious provide a patentable distinction? The Federal Circuit held that it did not, stating that "[e]ven if no prior art of record explicitly discusses the [limitation], the . . . application itself instructs that [the limitation] is not an additional requirement imposed by the claims on the [claimed invention], but rather a property necessarily present in the [claimed invention]." *In re Kubin*, 561 F.3d 1351, 1357-58, 90 USPQ2d 1417, 1422 (Fed. Cir. 2009) (as quoted in *Par Pharm., Inc. v Twi Pharm., Inc.*, 773 F.3d 1186, 112 USPQ2d 1945, 1952 (Fed. Cir. 2014).

In *KSR*, the Supreme Court held that:

> When a work is available in one field of endeavor, design incentives and other market forces can prompt variations of it, either in the same field or a different one. If a person of ordinary skill can implement a predictable variation, § 103 likely bars its patentability. For the same reason, if a technique has been used to improve one device, and a person of ordinary skill in the art would recognize that it would improve similar devices in the same way, using the technique is obvious unless its actual application is beyond his or her skill.

KSR Int'l Co. v. Teleflex Inc., 550 US 398, 417, 82 USPQ2d 1385, 1396 (2007). Given the teachings above, creation of a potato line as broadly as claimed in claim 1 is obvious in view of the above references.

Regarding the Markush group of added disease resistance traits and genes, Bradshaw teaches that "[m]ost potato-breeding programs will have disease and pest

resistance as an objective." Bradshaw, p. 173. Bradshaw further teaches using the H1 gene against G. rostochiensis and S. demissum-derived R-genes (Id.), and thus adding a disease-resistance trait, such as the above, to the claimed potato line is also obvious.

Thus, claims 1-3 and 18 are obvious. Given the well-known yield benefits in tetraploid potatoes, it would have been obvious to obtain a tetraploid plant (see, e.g., Jansky, p. 35), and thus claim 4 is obvious.

Maris, on pages 16 & 17, for example, teaches all` the steps of the method of claims 5 and 8, which, in any case, are routine breeding steps. Thus claims 5 and 7-8 are obvious. The plants and plant parts obtained by such a method are also obvious, and so claims 9-13 and 16-17 are obvious.

Jansky teaches that tissue culture of potatoes is routine, and regenerating plants from the tissue culture is also routine (e.g., Jansky, p. 39), and thus claims 14-15 are obvious.

Double Patenting

The nonstatutory double patenting rejection is based on a judicially created doctrine grounded in public policy (a policy reflected in the statute) so as to prevent the unjustified or improper timewise extension of the "right to exclude" granted by a patent and to prevent possible harassment by multiple assignees. A nonstatutory obviousness-type double patenting rejection is appropriate where the conflicting claims are not identical, but at least one examined application claim is not patentably distinct from the reference claim(s) because the examined application claim is either anticipated by, or would have been obvious over, the reference claim(s). See, e.g., *In re Berg*, 140 F.3d 1428, 46 USPQ2d 1226 (Fed. Cir. 1998); *In re Goodman*, 11 F.3d 1046, 29 USPQ2d 2010 (Fed. Cir. 1993); *In re Longi*, 759 F.2d 887, 225 USPQ 645 (Fed. Cir. 1985); *In re Van Ornum*, 686 F.2d 937, 214 USPQ 761 (CCPA 1982); *In re Vogel*, 422

F.2d 438, 164 USPQ 619 (CCPA 1970); and *In re Thorington*, 418 F.2d 528, 163 USPQ 644 (CCPA 1969).

A timely filed terminal disclaimer in compliance with 37 CFR 1.321(c) or 1.321(d) may be used to overcome an actual or provisional rejection based on a nonstatutory double patenting ground provided the conflicting application or patent either is shown to be commonly owned with this application, or claims an invention made as a result of activities undertaken within the scope of a joint research agreement.

Effective January 1, 1994, a registered attorney or agent of record may sign a terminal disclaimer. A terminal disclaimer signed by the assignee must fully comply with 37 CFR 3.73(b).

The USPTO internet Web site contains terminal disclaimer forms which may be used. Please visit http://www.uspto.gov/forms/. The filing date of the application will determine what form should be used. A web-based eTerminal Disclaimer may be filled out completely online using web-screens. An eTerminal Disclaimer that meets all requirements is auto-processed and approved immediately upon submission. For more information about eTerminal Disclaimers, refer to http://www.uspto.gov/patents/process/file/efs/guidance/eTD-info-I.jsp.

11. Claims 1-18 are provisionally rejected on the ground of nonstatutory obviousness-type double patenting as being unpatentable over claims 3 and 9 of copending Application No. 13/504,156. Although the conflicting claims are not identical, they are not patentably distinct from each other. This is a provisional double patenting rejection since the conflicting claims have not yet been patented.

Claims 3 and 9 of the 13/504,156 application relate to potato lines/plants of a “diploid, fertile, self-compatible and essentially homozygous potato line” comprising agronomically desirable traits and methods using such lines to create plants comprising agronomically desirable traits. The specifications of the two applications teach the same deposited potato lines. Claims 3 and 9 of the 13/504,156 application claim a broad genus, including, e.g., pest/disease resistance, while the instant claim set

encompass a sub-genus of the claimed genus listing numerous specific examples of genes associated with, e.g., pest/disease resistance.

Conclusion

12. No claim is allowed.

Allowable Subject Matter

13. The subject matter of claim 6 is free of the prior art made of record.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to RUSSELL BOGGS whose telephone number is (571)272-2805. The examiner can normally be reached on Monday through Friday between 8:00 AM to 6:30 PM (Mountain Time).

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Amjad Abraham, can be reached at 571-270-7058. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov. Should

you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free). If you would like assistance from a USPTO Customer Service Representative or access to the automated information system, call 800-786-9199 (IN USA OR CANADA) or 571-272-1000.

/RUSSELL BOGGS/
Examiner, Art Unit 1663

Request for Information under 37 CFR § 1.105

Applicant and the assignee of this application are required under 37 CFR § 1.105 to provide the following information that the examiner has determined is reasonably necessary to the examination of this application.

This request is being made for the following reasons:

Applicant is claiming a seed of plant variety 'AGVD1 (NCIMB 41663), AGVD2 (NCIMB 41664), AGVD3 (NCIMB 41665) and AGVD17 (NCIMB 41765)', but the instant specification is silent about what starting materials and methods were used to produce plant variety 'AGVD1 (NCIMB 41663), AGVD2 (NCIMB 41664), AGVD3 (NCIMB 41665) and AGVD17 (NCIMB 41765).' The requested information is required to make a meaningful and complete search of the prior art.

In response to this requirement, if known, please provide answers to each of the following interrogatories eliciting factual information:

(i) Please supply the breeding methodology and history regarding the development of the instant variety.

a) Such information should include the all the designations / denominations used for the original parental lines.

b) Information should also include information pertaining to the public availability of the original parental lines.

c) The breeding method used should be set forth such as whether single seed descent, bulk method, backcross method, or some other method was used.

d) The filial generation in which the instant plant was chosen should be set forth.

e) Information pertaining to the homozygosity or heterozygosity of the parents as well as the instant plant should be set forth.

f) Are there any patent applications or patents in which sibs of the instant plant are claimed? If so, please set forth serial numbers and names of the sibs.

If Applicant views any or all of the above requested information as a Trade Secret, then Applicant should follow the guidance of MPEP § 724.02 when submitting the requested information.

In responding to those requirements that require copies of documents, where the document is a bound text or a single article over 50 pages, the requirement may be met by providing copies of those pages that provide the particular subject matter indicated in

the requirement, or where such subject matter is not indicated, the subject matter found in applicant's disclosure. Please indicate where the relevant information can be found.

The fee and certification requirements of 37 CFR § 1.97 are waived for those documents submitted in reply to this requirement. This waiver extends only to those documents within the scope of this requirement under 37 CFR § 1.105 that are included in the applicant's first complete communication responding to this requirement. Any supplemental replies subsequent to the first communication responding to this requirement and any information disclosures beyond the scope of this requirement under 37 CFR § 1.105 are subject to the fee and certification requirements of 37 CFR § 1.97.

The applicant is reminded that the reply to this requirement must be made with candor and good faith under 37 CFR § 1.56. Where the applicant does not have or cannot readily obtain an item of required information, a statement that the item is unknown or cannot be readily obtained may be accepted as a complete reply to the requirement for that item.

This requirement is an attachment of the enclosed Office action. A complete reply to the enclosed Office action must include a complete reply to this requirement. The time period for reply to this requirement coincides with the time period for reply to the enclosed Office action.

/AMJAD ABRAHAM/
Supervisory Patent Examiner, Art Unit 1663

I hereby certify that this paper (along with any paper referred to as being attached or enclosed) is being transmitted via the Office electronic filing system in accordance with 37 CFR § 1.6(a)(4).

Dated:September 14, 2016 Signature: /Sarah Walsh/
(Sarah Walsh)

Docket No.: 091876-0027 **PATENT**

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of: Willem Hendrik LINDHOUT, et al.	:	Customer Number: 23630
Application No.: 14/112,747	:	Confirmation No.: 5919
Filed: December 23, 2013	:	Art Unit: 1663
For: Hybrid Seed Potato Breeding	:	Examiner: Russell T. BOGGS

Mail Stop – Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

AMENDMENT IN RESPONSE TO NON-FINAL OFFICE ACTION UNDER 37 C.F.R. 1.111

MS Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

INTRODUCTORY COMMENTS

In response to the Office Action dated March 14, 2016, please amend the above-identified U.S. as directed herein. A Petition for Extension of Time (3 months), a Statement relating to deposited biological material, a response to information requested under 37 CFR 1.105 and an Information Disclosure Statement are concurrently filed herewith. A copy of the Declaration under 37 CFR 1.132 and expert testimonies filed in the copending Application No. 13/504, 156 are also submitted concurrently herewith. Reconsideration and further examination of the present application is requested in light of the following

Amendments to the Specification begin on Page 2.
Amendments to the Claims begin on Page 5.
Remarks/Arguments begin on Page 11.

AMENDMENTS TO THE SPECIFICATION

In the following amendment to the specification, ~~deletions are shown with strike through~~, and additions are underlined.

Please amend the paragraph at page 9, lines 15-21 as follows:

Proper (commercial) sources of tetraploid cultivars are provided through the European Cultivated Potato Database (ECPD) at ~~http://~~www.europotato.org/menu.php and/or the Potato Pedigree Database of the expertise group Plant Breeding (a cooperation between the Laboratory of Plant breeding of Wageningen UR, the Netherlands) and the business unit Biodiversity and breeding of Plant Research International (PRI), Wageningen, the Netherlands at ~~http://~~www.plantbreeding.wur.nl/potatopedigree/.

Please amend the paragraph at page 34, lines 19-30 as follows:

Plants according to the present invention include the line with breeders reference AGVD1, representative seeds of said line having been deposited on 23 October 2009 with the NCIMB, Ferguson Building, Craibstone Estate, Bucksburn, Aberdeen, AB219YA, Scotland under accession number NCIMB 41663 and breeders reference AGVD1, which plant represents an F2 obtained by crossing a plant of parent IVP07-1001/4 (see Example 1), which is an essentially homozygous, diploid, fertile and self-compatible but non-vigorous potato line with a plant of parent D1, which is an elite diploid breeding clone that is essentially non-homozygous, and is not self-compatible, but which has the desirable agronomic trait of yellow flesh color and exhibits good cooking quality in that the flesh is not greying after cooking, and selfing the resulting F1 offspring plants. It is estimated that AGVD1 is about 50% homozygous, meaning that about 50% of the loci for which the parents differ are homozygous.

Please amend the paragraph bridging pages 34 to 35 as follows:

Plants according to the present invention also include the line with breeders reference AGVD2, representative seeds of said line having been deposited on 23 October 2009 with the NCIMB, Ferguson Building, Craibstone Estate, Bucksburn, Aberdeen, AB219YA, Scotland under

accession number NCIMB 41664 and under breeders reference AGVD2, which line represents an (F1 x F1) line (or pseudo F2) obtained by crossing a plant of parent IVP07-1001/4 as indicated above with a plant of parent D1 as indicated above, and mutually crossing individual F1 offspring plants, that share the IVP07-1001/4 parent but have different individuals of the D1parent. It is estimated that AGVD2 is about 25% homozygous for the loci for which the parents differ.

Please amend the paragraph at page 35, lines 7-14 as follows:

Plants according to the present invention also include the line with breeders reference AGVD3, representative seeds of said line having been deposited on 23 October 2009 with the NCIMB, Ferguson Building, Craibstone Estate, Bucksburn, Aberdeen, AB219YA, Scotland under accession number NCIMB 41665 and under breeders reference AGVD3, which line represents a BC1 population obtained by crossing a plant of parent IVP07-1001/4 as indicated above with a plant of parent D1 as indicated above, and backcrossing the resulting F1 offspring plants with the IVP07-1001/4 parent. It is estimated that AGVD3 is about 75% homozygous for the loci for which the parents differ.

Please amend the paragraph at page 35, lines 15-23 as follows:

Plants according to the present invention also include the line with breeders reference AGVD17, representative seeds of said line having been deposited on 5 October 2010 with the NCIMB, Ferguson Building, Craibstone Estate, Bucksburn, Aberdeen, AB219YA, Scotland under accession number NCIMB 41765 and under breeders reference AGVD17, which line represents an F3 population obtained by crossing a plant of parent IVP07-1001/4 as indicated above with a plant of parent D2 as indicated above, and backcrossing the resulting F1 offspring plants with the IVP07-1001/4 parent, followed by two steps of selfing. It is estimated that AGVD17 is > 80% homozygous for the loci for which the parents differ.

Please amend the paragraph bridging pages 52 to 53 as follows:

Though twenty-five sequences did not meet the criteria described above, they were kept as these were were known to be highly informative in theGoldenGate SNP assays. These SNPs even allowed to discriminate between the five genotype classes that occur in tetraploids (Voorrips et al,

EAPR-EUCARPIA congress "Potato Breeding after completion of the DNA Sequence of the Potato Genome", 27-30 June 2010, Wageningen, the Netherlands, e-publication at ~~http://~~edepot.wur.nl/143559, pg 42).

Please amend the paragraph at page 75, lines 7-20 as follows:

Hence, the present invention relates to hybrid plants resulting from a cross between two essentially homozygous parental breeding lines, at least one of which has incorporated in its genome, and provides to the F1 hybrid, resistance to *Phytophthera infestans* from *Solanum bulbocastanum* or *Solanum demissum*, Resistance to *G. rostochiensis* Ro1 from *Solanum tuberosum* subsp. *andigena* CPC 1690 or 1673, resistance to *G. pallida* from *Solanum tuberosum* subsp. *andigena*, resistance to *Globodera rostochiensis* Ro5-22 and *G. pallida* Pa2-D383 from *Solanum tuberosum* accession 78-3778, resistance to *G. pallida* Pa2 and Pa3 ~~VAN WELKE ACCESSIE ????,~~ VAN WELKE ACCESSIE, resistance to *G. pallida* Pa3 from *Solanum tuberosum* accession 3704-76, resistance to *M. chitwoodi* from *Solanum bulbocastanum*, resistance to *M. chitwoodi/M. fallax* from *Solanum fendlerii* and *Solanum hougassii*, Resistance to *M. hapla* from *Solanum chacoense* and *Solanum tarijense*, Resistance to PVX from *Solanum tuberosum* cv Pentland Ivory, and resistance to PVS from *Solanum tuberosum* DW 83-3121.

Please amend the paragraph at page 95, lines 1-3 as follows:

15. Draaistra, J. (2006). Genetic analysis of root-knot nematode resistance in potato. PhD thesis Wageningen University, The Netherlands. ~~http://~~edpot.wur.nl/43902

AMENDMENTS TO THE CLAIMS

1. (Currently Amended) A diploid, fertile, self-compatible and essentially homozygous potato line comprising plants having an average tuber yield expressed in grams of fresh weight of at least 200 grams per plant, wherein said line in addition to said tuber yield comprises at least one, more preferably at least 2, 3, 4, 5 or 6 agronomically desirable traits or genes, wherein said self-compatibility originates from *Solanum chacoense* as present in a plant of potato lines NCIMB accession number 41663, NCIMB accession number 41664, NCIMB accession number 41665, or NCIMB accession number 41765, representative seeds of said lines having been deposited with the NCIMB, Aberdeen, Scotland under breeders reference AGVD1, AGVD2, AGVD3, and AGVD17 respectively, and wherein said trait or gene is selected from the group consisting of resistance to *Phytophthora infestans* from *Solanum bulbocastanum* or *S. demissum*, resistance to *G. rostochiensis* Ro1 from *S. tuberosum* subsp. *andigena* CPC 1690 or 1673, resistance to *G. pallida* from *S. tuberosum* subsp. *andigena,* resistance to *G. rostochiensis* Ro5-22 and *G. pallida* Pa2-D383 from *S. tuberosum* accession 78-3778, resistance to *G. pallida* Pa2 and Pa3 from ~~Solanum spegazzinii~~ *Solanum spegazzinii* and ~~Solanum tarijense~~ *Solanum tarijense*, resistance to *G. pallida* Pa3 from *S. tuberosum* accession 3704-76, resistance to *M. chitwoodi* from *S. bulbocastanum*, resistance to *M. chitwoodi/M. fallax* from *S. fendlerii* and *S. hougassii*, resistance to *M. hapla* from *S. chacoense* and *S. tarijense*, resistance to PVX from *S. tuberosum* cv Pentland Ivory, ~~and~~ resistance to PVS from *S. tuberosum* DW 83-3121, ~~the~~ beta-carotene hydroxylase 2 (CHY2) gene, ~~the~~ zeaxanthin epoxidase (ZEP) gene, ~~the~~ potato gene for granule-bound starch synthase, ~~the~~ gene for beta-fructofuranosidase or potato acid invertase, ~~the~~ sucrose-phosphate-synthase gene, a gene for the putative invertase inhibitor precursor (INH1/INH2), ~~the~~ gene for tuber polyphenol oxidase PPO, ~~the~~ sucrose synthase gene, ~~the~~ chlorogenate inducer gene, ~~the~~ gene defined by expressed sequence tags EST724115 and EST717227 for asparagine synthetase, ~~the~~ phosphorylase-L gene, ~~the~~ gene for R1 protein or glucan water dikinase, all from *S. tuberosum*, ~~the~~ *S. demissum* R2 and R2-like protein genes, ~~the~~ *S. hjertingii* R2 late blight resistance protein (Rpi-hjt1.3) gene, ~~the~~ ABPT Rpi protein gene, ~~the~~ *S. venturii* late blight resistance protein genes, ~~the~~ *S. bulbocastanum* putative disease resistant protein RGA2 gene, ~~the~~ *S. bulbocastanum* blight resistance protein RPI gene, ~~the~~ *S. bulbocastanum* late

blight resistance protein Rpi-blb2 (Rpi-blb2) gene, ~~the~~ *S. bulbocastanum* NBS-LRR resistance protein (Rpi-bt1) gene, ~~the~~ *S. tuberosum* nematode resistance protein (Gro1-4) gene, ~~the~~ *S. tuberosum* disease resistance protein Gpa2 gene, ~~the~~ *S. tuberosum* subsp. *andigena* ADG2/2x(v-2)7a gene controlling resistance to potato Y and A potyviruses, ~~and/or~~ ~~the~~ *S. tuberosum* subsp. *andigena* ry-1 gene for resistance gene-like, exons 1-6, splice variants C38 and C19, ~~the~~ dihydroflavonol 4-reductase gene, ~~the~~ flavonoid 3',5'-hydroxylase gene and ~~the~~ gene for the R2R3 MYB transcriptional regulator of anthocyanin-1 production pathway all from *S. tuberosum L.*, ~~the~~ *S. demissum* late blight resistance protein (R1) gene, ~~the~~ potato late blight resistance protein R3a gene from *S. tuberosum L.*, and ~~the~~ *S. bulbocastanum* NBS-LRR resistance protein (Rpi-bt1) gene.

2. (Currently Amended) A hybrid ~~Hybrid~~ potato seed resulting from a cross between two essentially homozygous parental potato breeding lines, at least one of which is a plant according to claim 1.

3. (Currently Amended) A hybrid ~~Hybrid~~ potato plant[[s]] grown from the seed of claim 2.

4. (Currently Amended) A tetraploid plant produced by doubling the genome of ~~the plant~~ plants of claim 1.

5. (Currently Amended) A method for producing a diploid, fertile, self-compatible and essentially homozygous potato line comprising plants having an average tuber yield expressed in grams of fresh weight of at least 200 grams per plant, comprising:

(a) providing a first potato plant, which first potato plant is a plant of a first diploid, self-compatible, fertile and essentially homozygous potato line, wherein said self-compatibility originates from *Solanum chacoense* as present in a plant of potato lines NCIMB accession number 41663, NCIMB accession number 41664, NCIMB accession number 41665, or NCIMB accession number 41765, representative seeds of said lines having been deposited with the NCIMB, Aberdeen, Scotland under breeders reference AGVD1, AGVD2, AGVD3, and AGVD17 respectively;

(b) providing a second potato plant, wherein said second potato plant is a plant of a diploid or tetraploid potato line which may have any level of homozygosity, said line of said second potato plant comprising plants having an average tuber yield expressed in grams of fresh weight of at least 200 grams per plant;

(c) cross pollinating said first and second potato plant to provide seeds and collecting said seeds to thereby provide an offspring generation in the form of a hybrid offspring seed;

(d) growing said hybrid offspring seed into a population of hybrid offspring potato plant and selecting from said population plants producing tubers at a yield expressed in grams of fresh weight of at least 200 grams per plant, and

(e) selfing said hybrid offspring potato plant or backcrossing said hybrid offspring potato plant to a potato plant of said line of said first potato plant, for between 1 to 8 selfings or backcrosses, to thereby provide a diploid, self-compatible and essentially homozygous potato breeding line comprising plants having an average tuber yield expressed in grams of fresh weight of at least 200 grams per plant

(f) said method further comprising the step of introducing into a plant of said diploid, self-compatible and essentially homozygous potato breeding line obtained in step (e) at least one trait or gene selected from the group consisting of resistance to *Phytophthora infestans* from *Solanum bulbocastanum* or *S. demissum*, resistance to *G. rostochiensis* Ro1 from *S. tuberosum* subsp. *andigena* CPC 1690 or 1673, resistance to *G. pallida* from *S. tuberosum* subsp. *andigena,* resistance to *G. rostochiensis* Ro5-22 and *G. pallida* Pa2-D383 from *S. tuberosum* accession 78-3778, resistance to *G. pallida* Pa2 and Pa3 from ~~Solanum spegazzinii~~ *Solanum spegazzinii* and ~~Solanum tarijense~~ *Solanum tarijense*, resistance to *G. pallida* Pa3 from *S. tuberosum* accession 3704-76, resistance to *M. chitwoodi* from *S. bulbocastanum*, resistance to *M. chitwoodi/M. fallax* from *S. fendlerii* and *S. hougassii*, resistance to *M. hapla* from *S. chacoense* and *S. tarijense*, resistance to PVX from *S. tuberosum* cv Pentland Ivory, ~~and~~ resistance to PVS from *S. tuberosum* DW 83-3121, ~~the~~ beta-carotene hydroxylase 2 (CHY2) gene, ~~the~~ zeaxanthin epoxidase (ZEP) gene, ~~the~~ potato gene for granule-bound starch synthase, ~~the~~ gene for beta-fructofuranosidase or potato acid invertase, ~~the~~ sucrose-phosphate-synthase gene, a gene for the putative invertase inhibitor precursor (INH1/INH2), ~~the~~ gene for tuber polyphenol oxidase PPO, ~~the~~ sucrose synthase gene, ~~the~~

chlorogenate inducer gene, ~~the~~ gene defined by expressed sequence tags EST724115 and EST717227 for asparagine synthetase, ~~the~~ phosphorylase-L gene, ~~the~~ gene for R1 protein or glucan water dikinase, all from *S. tuberosum*, ~~the~~ *S. demissum* R2 and R2-like protein genes, ~~the~~ *S. hjer*tingii R2 late blight resistance protein (Rpi-hjt1.3) gene, ~~the~~ ABPT Rpi protein gene, ~~the~~ *S. venturii* late blight resistance protein genes, ~~the~~ *S. bulbocastanum* putative disease resistant protein RGA2 gene, ~~the~~ *S. bulbocastanum* blight resistance protein RPI gene, ~~the~~ *S. bulbocastanum* late blight resistance protein Rpi-blb2 (Rpi-blb2) gene, ~~the~~ *S. bulbocastanum* NBS-LRR resistance protein (Rpi-bt1) gene, ~~the~~ *S. tuberosum* nematode resistance protein (Gro1-4) gene, ~~the~~ *S. tuberosum* disease resistance protein Gpa2 gene, ~~the~~ *S. tuberosum* subsp. *andigena* ADG2/2x(v-2)7a gene controlling resistance to potato Y and A potyviruses, - ~~the~~ *S. tuberosum* subsp. andigena ry-1 gene for resistance gene-like, exons 1-6, splice variants C38 and C19, ~~the~~ dihydroflavonol 4-reductase gene, ~~the~~ flavonoid 3',5'-hydroxylase gene and ~~the~~ gene for the R2R3 MYB transcriptional regulator of anthocyanin-1 production pathway all from *S. tuberosum L.*, ~~the~~ *S. demissum* late blight resistance protein (R1) gene, ~~the~~ potato late blight resistance protein R3a gene from *S. tuberosum L.*, and ~~the~~ *S. bulbocastanum* NBS-LRR resistance protein (Rpi-bt1) gene.

6. (Currently Amended) The method of claim 5, wherein said first potato plant is a plant of potato line NCIMB accession number 41663, NCIMB accession number 41664, NCIMB accession number 41665, or NCIMB accession number 41765, representative seeds of said line having been deposited with the NCIMB, Aberdeen, Scotland under breeders reference AGVD1, AGVD2, AGVD3, and AGVD17 respectively.

7. (Currently Amended) A diploid potato breeding line~~, more preferably an elite line, obtainable~~ obtained by the method of claim 5.

8. (Currently Amended) A method for producing a uniform hybrid potato seed, comprising:

(a) providing a plant according to claim 1,

(b) providing a second potato plant, wherein said second potato plant is a plant of a diploid, fertile, self-compatible and essentially homozygous potato line, and

(c) cross pollinating said first and second potato plant and allowing one of ~~said parent plants~~ either said first or second potato plant to produce berries with seeds and collecting said seeds from said berries to provide an offspring generation in the form of a hybrid offspring seed.

9. (Original) A seed produced by the method of claim 8.

10. (Original) A hybrid potato plant, or part thereof, produced by growing the seed of claim 9.

11. (Original) Pollen or an ovule of the plant of claim 10.

12. (Original) A tuber from the plant of claim 10.

13. (Original) A potato plant having all of the physiological and morphological characteristics of the plant of claim 10.

14. (Previously Presented) A tissue culture of the plant of claim 10.

15. (Original) A potato plant regenerated from the tissue culture of claim 14, wherein said regenerated potato plant has all the physiological and morphological characteristics of a potato plant of claim 10.

16. (Original) A method of producing a hybrid potato plant, said method comprising producing a seed according to the method of claim 8 and growing said seed into a potato plant.

17. (Original) A potato plant produced by the method of claim 16, or a tuber thereof.

18. (Original) A set of at least 2 intercrossable diploid, fertile, self-compatible and essentially homozygous potato lines comprising plants according to claim 1.

19. (New) An elite diploid potato breeding line obtained by the method of claim 5.

REMARKS/ARGUMENTS

Status of Claims

Claims 1-18 were pending in this application. Claims 1-8 have been amended. Claim 19 is newly added.

Basis for this amendment is present throughout the application as filed. For example, support for the amendment to claims 1 and 5 can be found at page 6, lines 10-12, at page 11, lines 6-12, at page 33, lines 32-33, at page 34, lines 1-2, page 39, lines 1-5, at page 48, lines 21 to page 26, and at Table 6.

Claims 1-8 have been amended to correct informalities. No new matter is believed to be added herein.

Support for new claim 18 can be found at page 10, lines 4-6, at page 36, lines 28-31 and at page 81, lines 23-27.

Specification

The Examiner has objected to the specification for containing embedded hyperlinks and for reciting a question in the middle of page 75.

The specification has been amended to correct informalities. The specification has been amended to delete the embedded hyperlink and/or other form of browser-executable code. The amendments do not add new matter.

The Examiner has further objected to the specification for listing non-patent literature in Table 10 on pages 84-90. Incorporation of essential material into the specification by reference to a publication or online database is improper.

Applicant has amended the specification to include the material incorporated by reference. The material inserted is the material previously incorporated by reference and as such, the amendment contains no new matter.

The Examiner has also objected to the specification for lacking the address of NCIMB on pages 34-35, where the specification provides information regarding the deposits.

The specification has been amended to insert the address of NCIMB on pages 34-35 where necessary.

Claim Objections

Claims 1-3 and 5-6 are objected to because of informalities.

A. Claims 1 and 5 are objected to for the following reasons: (1) "Solanum spegazzinii" and "Solanum tarijense" are not italicized, (2) the recitation of the word "and", and (3) the recitation of the phrase "the expressed sequence tags EST724115 and EST717227 for aspargine synthetase"

Claim 1 is amended to provide italicized type where necessary.

Claims 1 and 5 have been amended to delete the word "and" in line 13 of claim 1 and line 11 of claim 5(f) to correct the grammar of the Markush groups.

Claims 1 and 5 have been amended to reference the gene defined by the expressed sequence tags EST724115 and EST717227.

B. Claims 2 and 3 are objected to because it refers to the subject composition in plural.

Claims 2 and 3 are amended to recite the word "seed" and "plant" in singular.

C. Claim 6 is objected to because the word "accession number" is missing.

Claim 6 is amended to include the word "accession number" between "NCIMB" and each of "41663", "41664", "41665" and "41765".

Reconsideration and withdrawal of objection are respectfully requested.

Rejections under 35 U.S.C. § 112, second paragraph

Claims 1-18 are rejected under 35 U.S.C. § 112 (b), second paragraph, as being indefinite, for failing to particularly point out and distinctly claim the subject matter which Applicant regards as the invention.

A. According to the Examiner, claims 1 and 5 are indefinite in their recitation of numerous terms, for example "*S. demissum* R2 and R2-like protein genes".

The specification has been amended to include the material improperly incorporated by reference in Table 10. As amended, the specification defines the terms used in claims 1 and 5.

B. According to the Examiner, claims 1 and 5 are indefinite because several terms lack antecedent basis.

Claims 1 and 5 have been amended to correct the lack of antecedent basis issue by deleting several "the" terms.

C. According to the Examiner, claims 1 and 5 are indefinite in the recitation of "the *S. tuberosum* subsp. *andigena* ADG212x(v-2)7a gene controlling resistance to potato and A potyviruses; and/or the *S. tuberosum* subsp. *andigena* ry-1 gene for resistance gene-like, exons 1-6, splice variants C38 and C19".

Claims 1 and 5 have been amended to delete the phrase "and/or."

D. According to the Examiner, claim 4 is indefinite in the recitation of "the plant.

For clarity purposes, the phrase "the plant" is amended to "plants" in claim 4.

E. According to the Examiner, claim 7 is indefinite in the recitation of "diploid potato breeding line", "more preferably an elite line" and "obtainable".

The phrase "more preferably an elite line" is deleted from claim 7. The phrase "obtainable" is replaced with the phrase "obtained".

F. According to the Examiner, claim 8 is indefinite in the recitation of "said parent plants", as it is unclear to which plant is being referenced.

The phrase "said parent plants" in claim 8 is amended to "either said first or second potato plant".

Reconsideration and withdrawal of rejection are respectfully requested.

Rejections under 35 U.S.C. § 112, first paragraph

A. Claims 1-6 and 7-18 are rejected under 35 U.S.C. 112(a) or 35 U.S.C. 112 (pre-AIA), first paragraph, as failing to comply with the written description requirement.

According to the Examiner, the claims are broadly drawn to a diploid, fertile, self-compatible and essentially homozygous potato line or a seed thereof, comprising plants that have an average tuber yield of at least 200 g/plant with at least one agronomically desirable trait. Method claim 5 is drawn to a method to produce such a line and a method for producing a uniform hybrid potato seed. According to the Examiner, the Applicant claims a large genus, whereas the instant application describes only four potato lines to use in the claimed methods that were created from the same line, the IVP07-1001/4 line.

Claims 1-8 have been amended and limited to the species *S. tuberosum*.

The claimed self-compatible *S.tuberosum* lines originates from *Solanum chacoense* as present in a plant of potato lines NCIMB accession number 41663, NCIMB accession number 41664, NCIMB accession number 41665, or NCIMB accession number 41765, representative seeds,of said lines having been deposited with the NCIMB, Aberdeen, Scotland under breeders reference AGVD1, AGVD2, AGVD3, and AGVD17 respectively.

AGVD1, AGVD2, AGVD3 and AGVD17 represent four examples of *S. tuberosum* having various levels of homozygosity. It is clear from the application as filed that the Applicant has demonstrated the methods that can be used to produce (1) a diploid potato breeding line, (2) hybrid potato seed and (3) an offspring of a diploid potato breeding line of the species of *Solanum tuberosum*.

Reconsideration and withdrawal of rejection are respectfully requested.

B. Claims 1-18 are rejected under 35 U.S.C. 112(a) or 35 U.S.C. 112 (pre-AIA), first paragraph, as failing to comply with the written description requirement.

According to the Examiner, claims 1 and 5 make reference to database accession numbers, EST724115 and EST717227 in the claims and the terms are defined in reference to Table 10 on pages 84-90. The Examiner alleges that the meaning and scope of the claims can only be understood by reference to the accession numbers and Table 10 and such incorporation is improper.

Applicant has amended the specification to include the material information that was improperly incorporated by reference, thereby making this rejection moot.

C. Claims 1-18 are rejected under 35 U.S. C. 112(a) or 35 U.S.C. 112 (pre-AIA), first paragraph, as failing to comply with the enablement requirement.

According to the Examiner, the specification does not disclose a repeatable process to obtain the plant and thus it is not apparent if the plant is readily available to the public.

Applicant asserts that the deposit of seeds was made under the Budapest Treaty. A Statement is submitted concurrently herewith by the Attorney of record stating that the instant invention will be readily available to the public upon the issuance of a patent.

Reconsideration and withdrawal of rejection are respectfully requested.

Rejections under 35 U.S.C. § 103

A. Claims 1-5 and 7-18 are rejected under pre-AIA 35 U.S.C. 103(a) as being unpatentable over Jansky ("Breeding, Genetics, and Cultivar Development", *Advances in Potato Chemistry and Technology*, ed. J. Singh & L. Kaur, pp. 27-62, June, 2009) in view of Phumichai et al. (*Genome* 48:977-84, 2005), in view of Maris (*Euphytica* 46:15-33, 1990), Simmonds (*Potato Res* 40:191-214, 1997), in further view of Watanabe et al. (*Breed Sci* 46:329-36, 1996) and Bradshaw (*New and Modern Methods of Potato Breeding* 2007, pp. 157-77).

The Examiner has concluded that it would have been obvious to a person of ordinary skill in the art to modify the teachings of the said prior art references to arrive at the claimed invention.

The rejection is principally based on the prior art disclosure (Jansky and Phumichai) that using *Sli gene* originating from *Solanum chacoense* can be used to attain high levels of homozygosity in potato plants and the inbred plants can be used in heterosis breeding and to produce true potato seed and from Simmonds disclosure that diploid potato plants were at that time underutilized, therefore providing motivation to cultivate them and that heterosis produces a "spectacular" increase in yield. The Examiner has cited Maris and Watanabe as allegedly disclosing the claimed agronomically desirable traits. The Examiner has cited Bradshaw as allegedly disclosing the claimed disease-resistance trait.

Applicant respectfully disagrees. Without acquiescing to the merits of the rejection and solely to expedite prosecution, claims 1-8 have been amended.

The Scope And Content Of The Prior Art

Prior art potato breeding methods:

The applicant herewith provides an overview of the prior art of potato breeding up to the priority date of the present application. In this, it will be emphasized that the skilled person would not arrive at the claimed subject-matter and that the steps of obtaining such a plant are not common in the breeding of cultivated or commercial potatoes of the species *Solanum tuberosum.*

Plant breeding can be divided into the "classical" methods of slowly enriching plant populations with beneficial characteristics and "hybrid" breeding, wherein homozygous parental inbred lines are developed and crossed to produce a commercial (F1) hybrid variety. In order to improve the F1 hybrid, the essentially homozygous parental lines may be improved by deliberate interbreeding (crossing) of closely or distantly related individuals to produce new germplasm whereby traits or genes from a donor line are introduced into the genetic background of a favored line with high agronomical or horticultural value.

Although hybrid breeding procedures are suitable and presently used for many crop species, for *Solanum tuberosum*, the current breeding process is not a hybrid breeding process, but a process of crossing highly heterozygous tetraploid parental lines and selecting from tens or hundreds of thousands of offspring plants the new and improved germplasm. This process is excessively labor intensive and time consuming as explained in the introductory part of the description of the application as filed. Still, potato breeding on a commercial level occurs in this way, primarily because hybrid breeding methods have proven unsuccessful (see below). Although a large number of new varieties have been developed, today's potato industry is still dominated by two potato varieties that were developed a century ago: Bintje (1904) in (parts of) Europe and Russet Burbank (1876) in North America. Methods of potato breeding have not changed much in the 20th century.

Hybrid breeding in *Solanum tuberosum* has not been possible up to the present invention. While at the tetraploid level the provision of homozygous inbred lines is prevented due to inbreeding depression (*see* introduction of Uijtewaal, Euphytica, 36: 745-753, 1987, IDS filed Mar 24, 2015), homozygous inbred lines have not been obtained at the diploid level due to both inbreeding depression and self-incompatibility (*see* Watanabe at introduction). However such essentially homozygous lines are pivotal in allowing the introduction of new traits into *S. tuberosum* germplasm. There are presently a large number of traits available for potato breeding, for instance for resistance to phytophtera, but such traits cannot be included in the present commercial germplasms.

Hence, there is a long felt need for improved methods of breeding *S. tuberosum*. One of the preferred, but unavailable, methods is that of hybrid breeding using essentially homozygous parental lines of *S. tuberosum*, also evidenced by the wish expressed in Jansky's publication, which was quickly downplayed as commercially unfeasible due to "dramatic reductions in fertility and vigor", while referring to Phumichai, both publications currently being used by the Examiner in the obviousness rejection. Jansky refers to sibling mating as an attractive alternative to hybrid breeding, thereby suggesting – and further establishing the prejudice argued herein below – to stay clear of hybrid breeding in *S. tuberosum*.

Inbreeding:

Apart from exhibiting the complex tetrasomic inheritance, *S. tuberosum* breeding also suffers from the inbreeding depression. One of the earliest publications on potato breeding is by Krantz (*see* Krantz, Technical Bulletin 25, Dec 1924, IDS filed Mar 24, 2015). This reference advocates the use of self-fertilization or inbreeding that is successfully used in other crops. Krantz summarizes as follows:

> "*Inbreeding studies on maize by East (5) and Shull (41, 42, 43, 44) suggested to these investigators the value of selection in self-fertilized lines of normally cross-pollinated crop as a means for their improvement. […]. In a vegetatively propagated crop such as the potato, in which the resultant heterozygous F1 individuals can be propagated indefinitely, the method would appear to be particularly appropriate. Furthermore, the almost total absence of desirable parents among our present potato varieties which produce viable pollen, makes it imperative to utilize some method to secure the parents necessary for a basis for potato breeding work.*" [*emphasis added, see* Krantz at pages 23-24 under the section titled "Selection in self-fertilized lines].

It is thus clear that in 1924, some 90 years ago, inbreeding in *S. tuberosum* was advocated as a valuable and necessary course, but that there were problems with producing fertile offspring. This is just one indication of the presence of a long felt need. In consecutive sections of his book chapter, Krantz describes the effect of the inbreeding strategy on heterozygosity, vigor and fertility, and discloses the following:

> *"Improved varieties that are dependable as pollen parents are a necessary requisite to any great improvement in the potato varieties of this country. Selection in self-fertilized lines is the best method of obtaining such varieties."* [*emphasis added, see* Krantz at page 29 under the section titled "Conclusions"].

Thus, in 1924 there was already a strong scientific motivation to use inbreeding in potato breeding methods. However, 60 years onward, Jackson et al. in 1984 were not able to report more than that "inbreeding in potatoes presents a considerable challenge, but one for which the potential rewards are great." (*see* Jackson, 1984, Report of the XXVIII Planning Conference, IDS filed Mar 24, 2015). Hence, despite the rewards and the suggestion of inbreeding strategies 60 years earlier, there were no such breeding results to report in 1984 due, at large, to inbreeding depression.

Tetraploid *S. tuberosum* is a natural reservoir for deleterious and lethal alleles. Although tolerated in tetraploids via dominance and epistatic interactions, this genetic burden causes inbreeding depression when homozygosity is increased (*see* Birhman, Genome, 43, 495-502, 2000, IDS filed

Mar 24, 2015). The exact mechanism of this phenomenon is not known, but it is considered that homozygous expression of deleterious genes upon inbreeding is the cause of the very weak and infertile plants of which the seedlings are addressed as "… having small, gnarly, absolutely worthless tubers" (*see* Krantz at page 24 under the section titled "Effect of inbreeding on heterzygosity").

Diploids:

Following the first description of genetically less complex diploid wild *Solanum* species (*S. chacoense, S, phureja, S. stenotomum*, etc.) and even diploid *S. tuberosum* clones in the late fifties (*see* Hougas, Am. Potato Journal, 35: 701-707, 1958, IDS filed Mar 24, 2015) the use of cultivated diploid Solanum species was implemented in potato breeding (*see* Haynes, International symposium on key problems and potentials for greater use of the potato in the developing world, 1972, IDS filed Mar 24, 2015).

Initially, potato dihaploids (2n = 2x = 24= diploid *S. tuberosum*) were envisioned as a tool to simplify the breeding of *S. tuberosum* cultivar production by reducing tetraploid germplasm to a diploid breeding level. Hougas already indicated in 1958 that such diploids are of interest to plant breeders primarily because they would present a rapid method for obtaining a high degree of homozygosity, and that the use of chromosome doubling in monoploids (haploids derived from diploid species) would result in completely homozygous individuals.

One should bear in mind, however, that, although not as a result from self-fertilization, these diploid *S. tuberosum* clones also suffer from inbreeding depression, due to the reduction of the tetraploid genome to the diploid level via haploidization (*see* Uijtewaal at pages 745-746 under the section titled "Introduction" and at pages 750-752, under the section titled "Discussion"). Such diploid *S. tuberosum* clones are weak and feeble plants, produce hardly any tubers (*see* Uijtewaal at page 750, Fig. 1) and cannot be selfed (*see* Uijtewaal at page 752, fourth paragraph), i.e., such dihaploids are self-incompatible. This self-incompatibility means that selfed plants do not form berries upon self-fertilization, or that berries do not contain viable seeds after selfing. Despite the fact that these diploid *S. tuberosum* breeding programs were proposed some 55 years ago by Hougas, they have

never resulted in vigorous fertile homozygous diploid *S. tuberosum* lines before the present invention due to self-incompatibility and inbreeding depression.

A second motive for the production of diploids was to acquire a 'genetic bridge' between the genomes of the various *Solanaceae* species. By this technique, ploidy barriers between cultivated *S. tuberosum* and wild *Solanum* species could be circumvented by reducing tetraploid *S. tuberosum* to the diploid level and crossing the resultant dihaploids to the wild diploid *Solanum* spp. In the background of this wild diploid *Solanum* spp material, the *S. tuberosum* genes can be recombined to a beneficial constitution. Novel germplasm is then incorporated back into tetraploid *S. tuberosum* breeding programs via sexual crosses between tetraploid *S. tuberosum* and colchicine-doubled *S. tuberosum* diploids, or through 4x-2x crosses utilizing unreduced gamete formation. This breeding strategy is indicated as "germplasm enhancement", which is basically a diploid breeding scheme using wild *Solanum* relatives to prepare a desired combination of genes, and then to elevate this back to the tetraploid level of cultivated *S. tuberosum*. This breeding strategy is also described by Maris, Hermundstad and Watanabe.

Watanabe is an example of such a potato breeding scheme through germplasm enhancement. The aim of the scientific research described in Watanabe is to use potato diploids as a genetic bridge to transfer resistance genes to tetraploid *S. tuberosum*. This is a two-step process whereby in the first step resistance genes are transferred from the wild donor species into diploid *S. tuberosum* by crossing and selections of the resistant diploid *S. tuberosum* in the progenies of such crosses. In the second step the resistance is transferred from resistant diploid *S. tuberosum* genotypes into tetraploid *S. tuberosum* by crossings via the production of unreduced gametes (2n) in the diploids. The resulting tetraploid plants can be further crossed with commercial tetraploid breeding germplasm to combine favourable agricultural traits from the tetraploids with the resistance gene originating from the wild *Solanum* species. Watanabe is not the first publication wherein germplasm enhancement is described. Hougas already expressed this idea in 1958 and combining useful genes for classic potato breeding through germplasm enhancement is a major line of research ever since, as indicated by Maris and Hermundstad. However, these *S. tuberosum* diploids are not essentially homozygous.

Homozygous diploids:

As indicated above under the section titled 'Diploids', one of the foreseen advantages of diploids (or dihaploids as they are also called for *S. tuberosum*) was that they could rapidly provide for a high degree of homozygosity.

Homozygous diploids of potato can in principle be obtained by one of two ways:

1. Doubling haploids (*see* Uijtewaal), wherein the haploids are for instance obtained by ovule culture or anther culture (*see* Jacobsen, Theor. Appl. Genet, 52: 119-123, 1978, IDS filed Mar 24, 2015), or

2. Repeated selfings using the *S. chacoense Sli* gene (*see* Phumichai).

Of the 2 methods above, only doubling of haploids (1) has successfully resulted in the production of homozygous diploids of *S. tuberosum*. As noted earlier, although some improvement in tuber yield could be obtained in the case of using doubled haploids (*see* Uijtewaal at page 750, Fig. 1 and page 746, Table 1), this is not nearly the 200 g/plant as present in a plant obtained by the method of claim 8, and, furthermore, these plants were essentially infertile (*see* Uijtewaal at page 752, third paragraph).

In contrast, the inbreeding technique reported by Phumichai for obtaining homozygous potatoes is NOT performed with *S. tuberosum*. The plants of Phumichai are not *S. tuberosum* plants but a mixture of the genomes of three wild relatives *S. chacoense, S, phureja, and S. stenotomum*. The *Sli* gene donor, clone F1-1, is also not an *S. tuberosum* plant but an interspecific hybrid between a highly inbred clone (S7) of *S. chacoense* and a cultivated diploid *S. phureja* clone (*see* Birhman). The F1 (S0) clone 94H87 is a cross between the *Sli* gene donor clone F1-1, and "cultivated diploid" 93H100 (*see* Phumichai at page 979, Fig. 1). Birhman discloses the following:

> "The family 93H100 consisted of plants raised from a mixture of seeds obtained by bulk-pollen pollinations among the cultivated diploid potatoes (the S. *stenotomum* – *S. phureja* mixed population)." [emphasis added, *see* Birhman at page 426, column 2].

Hence, clone 93H100 is *S. stenotomum/S. phureja*, while clone F1-1 is *S. chacoense/S. phureja*.

The schematic figure herein below summarizes the *Sli* gene ancestry, and position of the 93H100 family described by Phumichai.

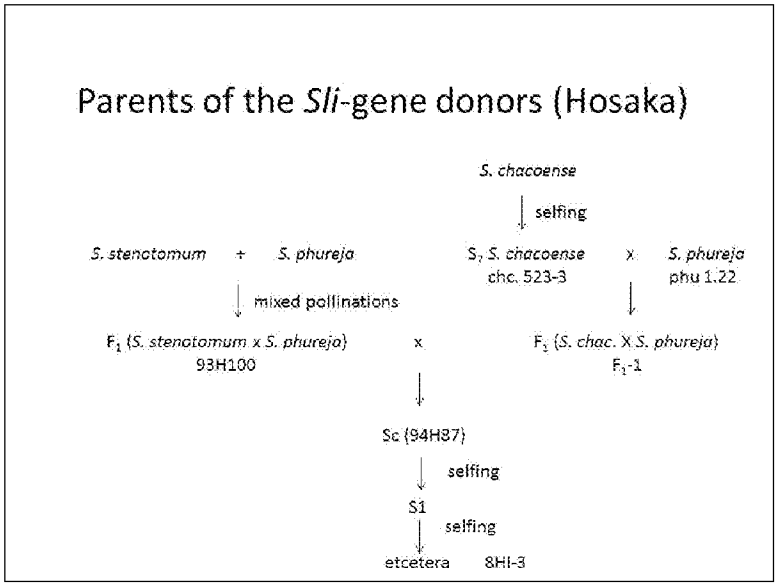


Fig. 1: Indication of the ancestry of the clones 93H100 (left), 94H87 (center) and F1-1 (right) (based on Birhman).

It should be noted that the term "Cultivated diploid potatoes" in Phumichai refers to *S. stenotomum/S. phureja* germplasm, and not to *S. tuberosum*. The term "cultivated diploid potatoes" must be understood in the light of Haynes, as follows:

> *"To date, consideration of breeding at the diploid level has always involved the derived diploids from Group Tuberosum. Other diploids have entered the picture primarily as sources of disease and insect resistance to be transferred largely through back cross programs. No systematic effort has been made to improve the diploid cultivars themselves through breeding."* [*see* Haynes at page 102, second paragraph].

The authors of Phumichai progress on that route, in an attempt to improve the germplasm of the diploid cultivated potatoes *S. stenotomum, S. chacoense* and *S. phureja*. Essentially, therefore, Phumichai is directed to enhancement of wild potato germplasm.

Differences Between the Prior Art and the Claimed Invention

The problem that the present inventors aimed to solve was to produce an essentially homozygous diploid potato plant (in the present case of the species *S. tuberosum*) that could be used as a true breeding parent for producing uniform hybrid potato seed. For that, the plant must show to be fertile, self-compatible and vigorous in that it produces more than 200 gram tubers.

Phumichai is directed to the same problem of attempting to produce highly homozygous diploid potato plants for producing TPS. This reference also uses the same method, namely inbreeding while overcoming the self-incompatibility problem using the *Sli gene*. The same problem is mentioned in Jansky (*see* Jansky at page 46, third paragraph), which for that matter explicitly refers back to Phumichai.

The difference between the present invention and Phumichai, or Jansky for that matter, is that the potato plant is a *Solanum tuberosum* plant, that is also vigorous in that it has a high (>200 gr/plant) tuber yield.

The present inventors arrived at such a plant by a method as presently claimed, in the first culturing steps encountering frail and weak plants with very few flowers and very few berries after self-pollination, and many berries without seed.

This phenomenon was already observed by Phumichai for the wild diploid cultivars when crossing a self-compatible potato plant having the *Sli gene* with cultivated plants raised from a mixture of seeds obtained by bulk-pollen pollinations among the cultivated diploid potatoes *S. stenotomum* and *S. phureja*.

Phumichai discloses that only 1 of 7 cross combinations reached S5 generation indicating serious loss of fertility and inbreeding depression reduced pollen viability and tuber yield (*see* Phumichai at page 982, second and third paragraphs).

Thus a skilled person would not be inclined to perform such a crossing with diploid plants of *Solanum tuberosum* since these tetraploid-derived diploids suffer from even more inbreeding depression.

Surprisingly, the inventors found that, successive rounds of selfing while selecting for plants that produce fertile offspring eventually resulted in plants with progressively improved fertility and vigour. Without wishing to be bound by theory, the inventors believe that this manner of selection indeed allowed the removal of a sufficient amount of deleterious genes from the *Solanum tuberosum* germplasm.

This result was fully unexpected, since selection for fertile plants has usually been associated with selecting for heterozygosity, not homozygosity.

> *"The slower than expected decline in heterozygosity has been reported by Brouwer and Osborn (1997) with inbreeding diploid alfalfa and by Scotti et al. (2000) with inbreeding tetraploid alfalfa. Consequential selection for vigor and fertility during selfing has been suggested as the cause (Brouwer and Osborn 1997). We previously suggested that reproductive traits, such as flowering versus nonflowering and the degree of pollen production, were strongly correlated with heterozygosity, and selection for fertility favored the more heterozygous plants."* [*see* Phumichai at page 982).

The present inventors were the first to follow the strategy that during the inbreeding process, the selection process of suitable offspring plants having an increased level of homozygosity should not be based on selecting vigorous plants having high tuber yields, but on selecting self-compatible plants having the highest level of fertility. For any potato breeder, this is a fully counterintuitive process. After all, potato breeders attempt to maximize the vigor of the plant and in particular the tuber yield while minimizing the amount of flowers and berries produced. After all, and in contrast to for instance tomato, the berries have no agronomic value, and cultivation of flowering and berries-producing potato plants may result in segregated seeds being shed that may grow into undesirable plants in commercial production fields. Hence, flowers, berries and viable seeds are not considered valuable and are selected against, while strong plants with high tuber yields are selected for further rounds of breeding.

The present inventors showed for the first time that an increase in vigor is possible when a sufficient level of fertility is attained, and that any premature selection for vigor results in ushering or selection for deleterious genes. Hence the inventors made a first cross between a diploid self-compatible potato line having the *Sli gene* and a diploid vigorous line of *S. tuberosum*, optionally backcrossed against the *Sli* donor, and continued the selfing process to increase the homozygosity while again selecting for fertility. In this manner, the inventors were surprisingly able to produce essentially homozygous inbred and diploid *S. tuberosum* plants that were fertile and vigorous.

Applicant wishes to provide further citations regarding no expectation of success, contrary to the Examiner's assertion.

Bachem (Unravelling the Potato Genome, 2008, IDS filed Mar 24, 2015) reported in 2008 on the perspectives of unraveling the potato genome on the FAO website dedicated to the International year of the Potato comments the following.

> *"Cultivated potato is what we call a highly heterozygous, self-incompatible outbreeder - in practice that makes it impossible to produce true breeding lines and so genetic improvement is a complex and lengthy process."* [emphasis added, *see* first paragraph of http://www.fao.org/potato-2008/en/perspectives/bachem.html]

Simko (Genetics, 173: 2237-2245, 2006, IDS filed Mar 24, 2015) recites Simmonds and classify potato as an outcrosser because it suffers from severe inbreeding depression that prevents development of homozygous lines. (*see* Simko at page 2237, right column).

Veilleux (Solcapnews 3(2): 4-5, 2011, IDS filed Mar 24, 2015) discloses the following:

> *"[…], the plant breeding community was captivated by the new opportunities to derive haploid and doubled haploid lines of commercial crops. Our intentions for applying the technique to the Haynes adapted phureja population of potato were motivated by the possibility of deriving homozygous lines as building blocks for hybrid true potato seed. My dissertation research at the University of Minnesota under the direction of Florian Lauer involved selecting 2n pollen producing clones from the adapted phureja population, crossing them to potato cultivars and evaluating the resulting tetraploid 4x-2x hybrids in the field. The high yield and cultivar like appearance of these 4x-2x hybrids was remarkable, despite the lack of selection on the phureja population for any traits except 2n pollen. Their heterogeneity could be attributed to genetic segregation expected to occur both during the formation of megasporocytes in the tetraploid cultivars as well as the formation of 2n pollen in the diploid phureja selections. We knew from the work of Wenzel in Germany and Uijtewaal in Holland that inbreeding depression in potato cultivars or (di)haploid breeding lines was severe, so that even if plants could be obtained, they were too weak to be of much use. However they had established a protocol for anther culture of potato. We hoped that the adapted phureja population would have greater tolerance to inbreeding".* [emphasis added; *see* Veilleux at page 4, first paragraph]

The above mentioned additional references also suggest that there was no expectation of success for inbreeding of *S. tuberosum* diploid material.

The general prejudice that production of homozygous true breeding lines of potato would not be possible is also indicated and supported with references on page 5, lines 1-28 of the application as filed.

Level Of Ordinary Skill In The Art

The person of ordinary skill in the art would have a background in potato breeding and crossing. The person of ordinary skill would be aware of the disclosures of the prior art documents discussed in this Amendment.

Objective Evidence Of Non-Obviousness

A Declaration by Willem Hendrik Lindhout under 37 CFR 1.132 and testimonies from the experts in the field of potato breeding were filed in the copending Application No. 13/504,156 on September 8, 2016 in response to the non-final Office Action mailed March 8, 2016.

A copy the declaration and testimonies are submitted herewith in order to provide additional information regarding the claimed invention that is probative of non-obviousness.

Commercial Importance of the invention

Applicant also wishes to bring the commercial and societal impact of the present invention, in establishing the background for non-obviousness. The Dutch company Solynta, which exploits the subject-matter of the present US patent application, had the honor to receive from the Dutch government the title of "National Icon 2014", for its groundbreaking technology in the field of potato breeding.

> *"For crops, the potato is fourth in terms of revenue in the world. Yet innovation in this crop has been silent for about 60 years. The variety 'bintje' is still the biggest variety in Europe. Solynta comes with a new breeding technique with potato seed instead of potatoes. The genetic material of the potato can be enhanced and so varied."* [emphasis added, *see* http://www.rvo.nl/nationaal-icoon-2014-hybride-aardappels].
>
> *"This groundbreaking technology makes it possible to increase the nutritional value of potatoes and to grow different types of potatoes". This means more efficient storage and transport and less disease. "Solynta underlines with this project the major Dutch contribution to the global world food supply."* [*see* http://www.rvo.nl/nationaal-icoon-2014-hybride-aardappels].

The Claimed Invention is Non-Obvious

The present invention solves the problem of producing diploid, homozygous inbred potato plants that are self-compatible, fertile and vigorous. The question to be answered is whether a skilled person would arrive at the claimed solution because of prior art teachings.

Applicant respectfully submits that this is not the case. If one would endeavor on a quest to find a vigorous homozygous Solanum tuberosum, this is not an endeavor one would engage on with any expectation of success.

If one of skill would choose to select the *Sli gene* donor F1-1 as taught by Phumichai and cross it with a cultivated diploid variety such as *S. phureja* or *S. stenotomum*, as described in Phumichai or Birhman, one would recognize that although some level of vigor may be obtained, it would not result in a fertile homozygote due to inbreeding depression, as clearly stated in Phumichai as follows:

> "*However, the fact that only 1 of 7 cross combinations reached S5 generation indicates that serious loss of fertility is typical in most families. Thus, inbreeding depression, typically expressed as reduced pollen viability and tuber yield ([…]), will certainly limit continued selfing of the present diploid inbreds*." [emphasis added; *see* Phumichai at page 982, left column, second paragraph].

Moreover, this line of development would result in a homozygous wild potato cultivar, not an *S. tuberosum* cultivar. The skilled person is also aware of the results of doubled haploid *S. tuberosum* experiments, and is aware that such breeding routes result in plants "too weak to be of much use" as stated herein above. Hence, the only experiment that resulted in essentially homozygous inbred potatoes with some tuber yield (not reported as being commercially relevant as the >200 gr/plant as presently claimed) were not obtained with dihaploids obtained from tetraploid *S. tuberosum*, but with naturally occurring diploid potato lines of the species *S. phureja* and *S. stenotomum*. Hence, if one would cross the *Sli* gene donor F1-1 from Phumichai or Birhman with a plant of a diploid *S. tuberosum* line, all one would expect is that this would not work.

Hence, performing a cross between a plant derived from *Sli* gene donor F1-1 from Phumichai or Birhman with a plant of a diploid *S. tuberosum* line with intent to produce a fertile and vigorous homozygous *S. tuberosum plant* will not lead to successful results and therefore not obvious to try, as results in that direction predicted failure.

Further, the Applicant notes that the combination of teachings of Phumichai and/or Jansky with Maris clearly reflects the hindsight that taints the present obviousness rejection. As argued hereinbefore, the main problem that underlies the invention is that it was hitherto impossible to produce essentially homozygous *Solanum tuberosum* plants that are vigorous in the sense as

presently claimed. The features of tuber yield and the level of homozygosity are therefore the essential elements that together define the invention.

In the context of obviousness, one cannot simply mosaic features together, especially not if a disclosure that is used to mosaic the invention expressly states its unsuitability for the purpose of the invention and the problem it solves.

In view of the foregoing, the claims are non-obvious. Reconsideration and withdrawal of the rejection are respectfully requested.

Double Patenting

Claims 1-18 are provisionally rejected on the ground of nonstatutory obviousness-type double patenting as being unpatentable over claims 3 and 9 of copending Application No. 13/504,156.

Applicants respectfully disagree.

Claims 3 and 9 of the copending Application No. 13/504,156 are drawn to a potato plant and methods of producing a diploid, fertile, self-compatible and essentially homozygous potato line of the species *Solanum tuberosum* or *Solanum tuberosum* comprising introgression segments of other tuber bearing *Solanum* species crossable to *Solanum tuberosum*, said potato line comprising a plant having a tuber yield expressed in grams of fresh weight of at least 200 grams; wherein at least 50% of loci in said plant are homozygous, wherein said self-compatibility originating from *Solanum chacoense* as present in a plant of potato lines NCIMB accession number 41663, NCIMB accession number 41664, NCIMB accession number 41665, or NCIMB accession number 41765, representative seeds of said lines having been deposited with the NCIMB, Aberdeen, Scotland under breeders reference AGVD1, AGVD2, AGVD3, and AGVD17 respectively, and wherein at least 25% of the plants of said line produce seed-carrying berries, wherein said plant comprises at least one agronomically desirable trait selected from the group consisting of insect tolerance, nematode resistance, disease resistance, herbicide tolerance, cold tolerance, drought tolerance, wet tolerance, tolerance to dry and wet rot, salinity tolerance, yellow flesh color and cold-sweetening resistance.

Amended claim 1 of the instant application is drawn a diploid, fertile, self-compatible and essentially homozygous potato line comprising an average tuber yield of at least 200 grams per

plant, wherein said line comprises at least one or more agronomically desirable traits or genes and wherein said self-compatibility originating from *Solanum chacoense* as present in a plant of potato lines NCIMB accession number 41663, NCIMB accession number 41664, NCIMB accession number 41665, or NCIMB accession number 41765, representative seeds of said lines having been deposited with the NCIMB, Aberdeen, Scotland under breeders reference AGVD1, AGVD2, AGVD3, and AGVD17 respectively.

An obviousness-type double patenting rejection is appropriate only when the claims of one application are obvious variants of the claims in another application or patent. The pending claims are not obvious variants because there is nothing in claims 1-9 of co-pending Application No. 14/382,952 that fairly suggests the subject matter of amended claim 1.

Reconsideration and withdrawal of the rejection are respectfully requested.

CONCLUSION

Should the Examiner be of the view that an interview would expedite consideration of the application, request is made that the Examiner telephone the applicant's attorney at (617) 535 4453 in order that any outstanding issues be resolved.

Applicant has included the fee for three-month extension of time fee. The Director is hereby authorized to charge any deficiency in the fees filed, asserted to be filed, or which should have been filed herewith (or with any paper hereafter filed in this application by this firm) to our Deposit Account No. 50-1133, under Order No. 091876-0027 from which the undersigned is authorized to draw.

Respectfully submitted,

McDERMOTT WILL & EMERY LLP

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Kristin A. Connarn
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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
14/112,747	12/23/2013	Willem Hendrik Lindhout	091876-0027	5919

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Washington, DC 20001

EXAMINER
BOGGS, RUSSELL T

ART UNIT	PAPER NUMBER
1663	

NOTIFICATION DATE	DELIVERY MODE
12/15/2016	ELECTRONIC

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

mweipdocket@mwe.com

Office Action Summary	Application No. 14/112,747	Applicant(s) LINDHOUT ET AL.	
	Examiner RUSSELL BOGGS	Art Unit 1663	AIA (First Inventor to File) Status No

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE *3* MONTHS FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☒ Responsive to communication(s) filed on *14 September 2016*.

☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on _____.

2a)☒ This action is **FINAL**. 2b)☐ This action is non-final.

3)☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on _____; the restriction requirement and election have been incorporated into this action.

4)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims*

5)☒ Claim(s) *1-19* is/are pending in the application.

5a) Of the above claim(s) _____ is/are withdrawn from consideration.

6)☐ Claim(s) _____ is/are allowed.

7)☒ Claim(s) *1-19* is/are rejected.

8)☐ Claim(s) _____ is/are objected to.

9)☐ Claim(s) _____ are subject to restriction and/or election requirement.

* If any claims have been determined allowable, you may be eligible to benefit from the **Patent Prosecution Highway** program at a participating intellectual property office for the corresponding application. For more information, please see http://www.uspto.gov/patents/init_events/pph/index.jsp or send an inquiry to PPHfeedback@uspto.gov.

Application Papers

10)☒ The specification is objected to by the Examiner.

11)☐ The drawing(s) filed on _____ is/are: a)☐ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

Priority under 35 U.S.C. § 119

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

Certified copies:

a)☐ All b)☐ Some** c)☐ None of the:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

** See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☒ Notice of References Cited (PTO-892)

2) ☒ Information Disclosure Statement(s) (PTO/SB/08a and/or PTO/SB/08b) Paper No(s)/Mail Date _____.

3) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date. _____ .

4) ☐ Other: _____.

DETAILED ACTION

The present application is being examined under the pre-AIA first-to-invent provisions.

Status

1. Claims 1-18, filed 18 October 2013, were examined and rejected in the Office action mailed 14 March 2016. Applicant responded on 14 September 2016 ("Response"). Claims 1-19 are examined herein.

Withdrawal of Objections and Rejections

2. The objections to the specification regarding 3 of the 4 hyperlinks, the four question marks on page 75 and the lack of an address on pages 34-35 are withdrawn in view of Applicant's amendments.

3. The objections to claims 1-3 and 5-6 are withdrawn in view of Applicant's amendments to the claims.

4. The rejection of claims 1 and 5 under 35 U.S.C. 112(b) or 35 U.S.C. 112 (pre-AIA), second paragraph, as being indefinite for lacking antecedent basis is withdrawn in view of Applicant's amendments to the claims.

5. The rejection of claims 1 and 5 under 35 U.S.C. 112(b) or 35 U.S.C. 112 (pre-AIA), second paragraph, as being indefinite because of the recitation of "and/or" in lines 25-28 of claim 1 and lines 24-26 of claim 5(f) is withdrawn in view of Applicant's amendments to the claims.

6. The rejection of claim 4 under 35 U.S.C. 112(b) or 35 U.S.C. 112 (pre-AIA), second paragraph, as being indefinite for lacking antecedent basis for "the plant" is withdrawn in view of Applicant's amendments to the claims.

7. The rejection of claim 7 under 35 U.S.C. 112(b) or 35 U.S.C. 112 (pre-AIA), second paragraph, as being indefinite regarding the recitation of "more preferably an elite line" and "obtainable" is withdrawn in view of Applicant's amendments to the claim.

8. The rejection of claim 8 under 35 U.S.C. 112(b) or 35 U.S.C. 112 (pre-AIA), second paragraph, as being indefinite for lacking antecedent basis for "said parent plants" is withdrawn in view of Applicant's amendments to the claims.

9. The rejection of claims 1-5 and 7-18 under pre-AIA 35 U.S.C. 103(a) is withdrawn in view of Applicant's amendments to the claims and the Lindhout declaration under 37 CFR § 1.132 with supporting letters.

Examiner's Note

10. Citations to the specification herein are abbreviated "Spec."

Specification

11. The disclosure is objected to because of the following informalities.

The disclosure is objected to because it contains an embedded hyperlink and/or other form of browser-executable code. Applicant is required to delete the embedded

hyperlink and/or other form of browser-executable code. See MPEP § 608.01. See page 53, line 19. All others were removed.

The specification lists non-patent literature in Table 10 on pages 84-90. Several of the claim terms in claims 1 and 5 are rejected under 35 USC 112(b) as being indefinite because they lack an art-accepted meaning. Applicant apparently intended to define the claim terms by reference to this table. It is the Office's interpretation that it is Applicant's clear intent to incorporate the essential material cited by reference to the non-patent literature into the claims. The meaning and scope of the claim limitations can only be understood by reference to listed references, however, such an incorporation is improper.

The incorporation of essential material into specification by reference to an unpublished U.S. application, foreign application or patent, to a publication, or to an online database is improper. Applicant is required to amend the specification to include the material incorporated by reference, if the material is relied upon to overcome any objection, rejection, or other requirement imposed by the Office. The amendment must be accompanied by a statement executed by the applicant, or a practitioner representing the applicant, stating that the material being inserted is the material previously incorporated by reference and that the amendment contains no new matter. 37 CFR 1.57(f).

Applicant's Arguments and Response

Applicant traverses the rejection in the paper filed 14 September 2016. On page 12 of the response, Applicant writes that the specification was amended to include the material improperly incorporated by reference."

Applicant's arguments have been fully considered but are not persuasive. No such amendment was made and furthermore, such an amendment would be improper under 37 C.F.R. 1.57(c), as stated above insofar since the material is essential material.

Appropriate correction is requested.

Claim Objections

12. Claims 2 and 4 are objected to because of the following informalities.

Claim 2 is objected to because the subject of claim 1 is a line, not a plant. Insertion of "of a line" between "plant" and "according" in line 2 would obviate this objection.

Claim 4 is objected to because it recites the plural "plants" in line 2 rather than reading on a single plant. It is preferable to direct a claim to a single invention because, for example, it avoids the question if a single plant would infringe the claim.

Appropriate correction is requested.

Claim Rejections - 35 USC § 112

The following is a quotation of 35 U.S.C. 112(b):

> (B) CONCLUSION.—The specification shall conclude with one or more claims particularly pointing out and distinctly claiming the subject matter which the inventor or a joint inventor regards as the invention.

The following is a quotation of 35 U.S.C. 112 (pre-AIA), second paragraph:

> The specification shall conclude with one or more claims particularly pointing out and distinctly claiming the subject matter which the applicant regards as his invention.

13. Claims 1-19 are rejected under 35 U.S.C. 112(b) or 35 U.S.C. 112 (pre-AIA), second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which the inventor or a joint inventor, or for pre-AIA the applicant regards as the invention.

Claims 1 and 5 are rejected because they recite numerous limitations, for example "*S. demissum* R2 and R2-like protein genes," in which genes are identified by a variety of terms where the meaning of the terms are indefinite. Terms such as "R2" and "R2-like" lack a general art-accepted meaning and Applicant does not explicitly define the terms in the specification. For example, there are two different "R2"s in the list below. Applicant provides Table 10 on pages 84 to 90 which cite to references from the literature, however, the incorporation of essential material in the specification by reference to an unpublished U.S. application, foreign application or patent, or to a publication is improper. 37 C.F.R. 1.57(c).

The terms in question include:

resistance to *Phytophthora infestans* from *Solarium bulbocastanum or S. demissum*, resistance to *G. rostochiensis* Ro1 from *S. tuberosum* subsp. *andigena* CPC 1690 or

1673,

resistance to *G. pallida* from *S. tuberosum* subsp. *andigena*,

resistance to *G. rostochiensis* Ro5-22 and *G. pallida* Pa2-D383 from *S. tuberosum*

accession 78-3778,

resistance to *G. pallida* Pa2 and Pa3 from *Solanum spegazzinii* and *Solanum tarijense*,

resistance to *G. pallida* Pa3 from *S. tuberosum* accession 3704-76,

resistance to *M. chitwoodi* from *S. bulbocastanum*,

resistance to *M. chitwoodi* / *M. fallax* from *S. fendlerii* and *S. hougassii*,

resistance to *M. hapla* from *S. chacoense* and *S. tarijense*,

resistance to PVX from *S. tuberosum* cv Pentland Ivory,

resistance to PVS from *S. tuberosum* DW 83-3121,

beta-carotene hydroxylase 2 (CHY2) gene;

zeaxanthin epoxidase (ZEP) gene;

potato gene for granule-bound starch synthase;

gene for beta-fructofuranosidase or potato acid invertase;

sucrose-phosphate-synthase gene;

gene for the putative invertase inhibitor precursor (INH1/INH2);

gene for tuber polyphenol oxidase PPO;

sucrose synthase gene;

chlorogenate inducer gene;

expressed sequence tags EST724115 and EST717227 for asparagine synthetase;

phosphorylase-L gene;

gene for R1 protein or glucan water dikinase, all from *S. tuberosum*;

S. demissum R2 and R2-like protein genes;

S. hjertingii R2 late blight resistance protein (Rpi-hjt1.3) gene;

ABPT Rpi protein gene;

S. venturii late blight resistance protein genes;

S. bulbocastanum putative disease resistant protein RGA2 gene;

S. bulbocastanum blight resistance protein RPI gene;

S. bulbocastanum late blight resistance protein Rpi-b1b2 (Rpi-blb2) gene;

S. bulbocastanum NBS-LRR resistance protein (Rpi-bt1) gene;

S. tuberosum nematode resistance protein (Gro1-4) gene;

S. tuberosum disease resistance protein Gpa2 gene;

S. tuberosum subsp. *andigena* ADG212x(v-2)7a gene controlling resistance to potato Y and A potyviruses;

S. tuberosum subsp. *andigena* ry-1 gene for resistance gene-like, exons 1-6, splice variants C38 and C19;

dihydroflavonol 4-reductase gene;

flavonoid 3′,5′-hydroxylase gene;

gene for the R2R3 MYB transcriptional regulator of anthocyanin-1 production pathway all from *S. tuberosum*

S. demissum late blight resistance protein (R1) gene;

potato late blight resistance protein R3a gene from *S. tuberosum L.*; and

S. bulbocastanum NBS-LRR resistance protein (Rpi-bt1) gene. Because of the

indefiniteness of these terms , the metes and bounds of the claims cannot be determined.

Furthermore, the meaning of these terms could arbitrarily change to designate something different during the lifetime of a patent. Thus, one's ability to determine the metes and bounds of the claim would be impaired. See *In re Hammack*, 427 F .2d 1378, 1382; 166 USPQ 204, 208 (CCPA 1970).

Applicant's Arguments and Response

Applicant traverses the rejection in the paper filed 14 September 2016. On page 13 of the response, Applicant writes that the "specification has been amended to include the material improperly incorporated by reference in Table 10."

Applicant's arguments have been fully considered but are not persuasive. No such amendment was made and furthermore, such an amendment would be improper under 37 C.F.R. 1.57(c), as stated above.

Dependent claims are included in this rejection because none provide further limitations obviating this rejection.

Claims 1 and 5 are rejected because they recite limitation the "*S. tuberosum* subsp. andigena ry-1 gene for resistance gene-like, exons 1-6, splice variants C38 and C19." The meaning of this claim limitation is indefinite. For example, why is "gene" recited twice?

Dependent claims are included in this rejection because none provide further limitations obviating this rejection.

Applicant did not specifically address this rejection.

The following is a quotation of 35 U.S.C. 112(a):

> (a) IN GENERAL.—The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same, and shall set forth the best mode contemplated by the inventor or joint inventor of carrying out the invention.

The following is a quotation of 35 U.S.C. 112 (pre-AIA), first paragraph:

> The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same and shall set forth the best mode contemplated by the inventor of carrying out his invention.

14. Claims 1-5 and 7-19 are rejected under 35 U.S.C. 112(a) or 35 U.S.C. 112 (pre-AIA), first paragraph, as failing to comply with the written description requirement. The claim(s) contains subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor or a joint inventor, or for pre-AIA the inventor(s), at the time the application was filed, had possession of the claimed invention.

Due to Applicant's amendments to the claims, the rejection is modified from the rejection set forth in the Office action mailed 14 March 2016 as applied to the above-referenced claims. Applicant traverses the rejection in the paper filed 14 September 2016. Applicant's arguments have been fully considered but are not persuasive.

Claim 1 is drawn to a diploid, fertile, self-compatible and essentially homozygous potato line having an average tuber yield expressed in grams of fresh weight of at least 200 grams per plant; wherein said line additionally comprises 1-6 agronomically desirable traits or genes selected from a Markush group and wherein the self-

compatibility originates from *S. chacoense* and is as present in a plant of potato lines NCIMB accession number 41663, NCIMB accession number 41664, NCIMB accession number 41665, or NCIMB accession number 41765, further where representative samples were deposited with the NCIMB under breeders reference AGVD1, AGVD2, AGVD3, and AGVD17.

Claims depending from claim 1 read on a hybrid potato seed produced from a cross between two essentially homozygous parental breeding lines and a potato plant(s) produced therefrom. Claim 8 reads on a method using a plant from claim 1. Claims 9-13 read on plants and plant parts produced by the method of claim 8. Claim 14 reads on a tissue culture produced by the plant of claim 10 and claim 15 reads on a plant produced from the tissue culture of claim 14. Claim 16 reads on a method using a seed produced according to claim 8 to produce a plant; claim 17 reads on a plant or tuber produced according to claim 16. Claim 18 reads on a set of at least 2 intercrossable diploid, fertile, self-compatible and essentially homozygous potato lines comprising plants according to claim 1.

Claim 5 is a method claim drawn to producing a diploid, fertile, self-compatible and essentially homozygous potato line starting with a first potato plant of a first diploid self-compatible, fertile and essentially homozygous potato line wherein the self-compatibility originates from *S. chacoense* and is as present in a plant of potato lines NCIMB accession number 41663, NCIMB accession number 41664, NCIMB accession number 41665, or NCIMB accession number 41765, further where representative samples were deposited with the NCIMB under breeders reference AGVD1, AGVD2,

AGVD3, and AGVD17 and crossing it to a second potato plant where the second potato plant has an average tuber yield expressed in grams of fresh weight of at least 200 grams per plant and then selfing or backcrossing to provide a diploid, self-compatible and essentially homozygous potato breeding line comprising plants having an average tuber yield of at least 200 grams per plant, and then introducing into a plant of said line at least one trait or gene selected from a Markush group.

Dependent claim 6 recites specific deposited cell lines; claim 7 encompasses a diploid potato breeding line obtained by the method of claim 5. Claim 19 reads on an elite diploid potato breeding line obtained by the method of claim 5.

To claim a genus under the written description requirement, Applicant is required to describe a representative number of species to reflect the variation within the genus or structures sufficient to define the genus. The factors to be considered include disclosure of complete or partial structure, physical and/or chemical properties, functional characteristics, structure/function correlation, methods of making the claimed product, or any combination thereof.

The claimed genera, in particular, include those set forth in the following exemplary claims:

claim 1 – any “diploid, fertile, self-compatible and essentially homozygous potato line” wherein the self-compatibility originates from *S. chacoense* and is as present in a plant of potato lines NCIMB accession number 41663, NCIMB accession number 41664, NCIMB accession number 41665, or NCIMB accession number 41765 together with at least one agronomically desirable trait;

claim 5 – any “diploid, fertile, self-compatible and essentially homozygous potato line” wherein the self-compatibility originates from *S. chacoense* and is as present in a plant of potato lines NCIMB accession number 41663, NCIMB accession number 41664, NCIMB accession number 41665, or NCIMB accession number 41765 as a starting material in part (a) to produce a “diploid, fertile, self-compatible and essentially homozygous potato line” with an added trait or gene.

The above claims, however, are not limited to the self-compatibility genes and/or potato lines described by the deposits. The claims encompass the genus of all self-compatibility that is “as present” in the deposited lines as well as other potato lines. Applicant fails to describe the scope of “as present.” For example, the meaning of "as" includes "for instance" and thus the claims also read on other embodiments of self-compatibility. Merriam-Webster, "as", accessed 2 December 2016.

Additionally, claim 13 represents a broad genus. Claim 13 requires all the physiological and morphological characteristics of the plant of claim 10, which is a plant produced by the method of claim 8, which starts with the plant of claim 1. Claim 1 recites only a handful of physiological and morphological characteristics – arguably just the tuber weight, and because claim 8 is a cross with another plant, the physiological and morphological characteristics which will be passed to any given offspring is uncertain and thus the plants produced by the method of claim 8 constitute a large genus.

In the specification, Applicant describes obtaining the IVP07-1001/4 line from publically available germplasm and plants that contain the *Sli* gene and using these plants to breed AGVD1, AGVD2, and AGVD3. Spec., pp. 46-47.

Applicant describes the deposited lines by breeders reference: AGVD1 (NCIMB 41663), AGVD2 (NCIMB 41664), AGVD3 (NCIMB 41665) and AGVD17 (NCIMB 41765). Id., pp. 34-35. Applicant describes a breeding process according to the instant invention starting with AGVD1-3 or AGVD17 on page 39 et seq.

Applicant describes crossing the *Sli* gene donor with various potato clones designated: IVP97-079-9, IVPAA-096-I8 and SH83-92-488 to generate clones D1, D2 and D3. Id., p. 49. Applicant describes creating a line containing the *Sli* gene with enhanced agricultural quality in Example 2, pp. 48-51. Applicant describes creating a molecular marker platform, i.e., SNPs, in Example 3, p. 51 et seq. In Example 4, Applicant describes the development of homozygous diploid potato plants and then F1 hybrid progeny, as well as subsequent generations. Id., p. 60 et seq. Applicant describes the meaning of the claim term “essentially homozygous” on pages 21-22 of the instant specification and suggests that the term reflects a level of homozygosity that is above 25% or 75%. Id., p. 21-22. Applicant generally describes adding an agronomically desirable trait to a potato genotype on, e.g. page 37.

Applicant fails to describe a representative number of species in comparison to the size of the genera claimed. In the instant application, Applicant describes only four potato lines to use in the claimed methods or as a starting point to obtain the claimed compositions, and all were created from the same line, the IVP07-1001/4 line.

Specification, pp. 46-48. Applicant fails to describe working examples of the broad genus of other potato lines, or embodiments of self-compatibility originating from *S. chacoense* falling within the scope of the claims. Further, Applicant only describes one parental line and fails to describe examples of claimed potato lines descended from different parental lines.

In the response filed on 14 September 2016, Applicant traverses the rejection under 35 USC 103 and provides background regarding the opinions of artisans in the relevant field. Response, pp. 16-22. In support of the argument, Applicant filed a declaration under 37 CFR 1.132 by Dr. Lindhout with supporting letters. Dr. Lindhout's declaration was formally filed in application serial no. 13/504,156, and the supporting letters were filed in European Application EP2699684 on 04 June 2014 and are copies therefrom. The letters establish the position that one of skill in the art does not believe Applicant to be in possession of the genus as broadly as claimed. For example, on page 2 of his letter, Dr. Koornneef states that

> Ever since Lindhout and his co-workers published their results in Potato Research volume 54 of 2011, showing high yielding hybrids from 87% homozygous F3 inbreds, I have been positively surprised that Solynta has managed to challenge this dogma. Solynta could successfully obtain essentially homozygous inbred lines by making use of genotypes that are selfcompatible and by choosing the right starting material that apparently is heterozygous for a lower number of deleterious alleles. This provides the basis of generating useful inbred lines that can become parents of F1 hybrids.

Letter from Koornneef (2012) p. 2 (underlining added). In another letter supplied in support of Dr. Lindhout's declaration, Dr. Jones states that

> "[I]n the mid 2000s we attempted to produce hybrid potato ourselves. . . . However, we never succeeded in developing any promising material in the

> form of fertile homozygotes that could be useful for hybrid breeding and that could tolerate recurrent selfing. . . . We considered that this was due to our inability to cross the Sli- self-compatibility locus into good enough or diverse enough diploid *Solanum* germplasm.

Letter from Dr. Jonathan D.G. Jones (2012), p. 1 (underlining added). Both letters applaud Applicant for finding potato lines suitable for the instant invention. Conversely, both letters teach that such lines are exceptional.

Applicant claims a large genus of potato plants and self-compatibility genes. Applicant fails to describe a representative number of species in comparison to the size of the genera claimed. Applicant also fails to describe any structure necessary and/or sufficient for a potato line to be used in the claims, for example the genes responsible for the self-compatibility or the DNA sequences thereof except by reference to the deposits; or the genes required for the potato lines. The specification therefore fails to provide an adequate written description to support the breadth of the claims. Applicant has not demonstrated possession to one of skill in the art of the instant invention as broadly as claimed at the time of filing, and one of skill in the art would not believe Applicant to be in possession of the genus as broadly as claimed, especially in view of the letters cited above.

Dependent claims are included in this rejection because none provide further limitations obviating this rejection.

Applicant's Arguments

Applicant argues against the rejection in the response filed on 14 September 2016. Response, p. 14. Applicant points out the claim amendments. *Id.* Applicant urges that "AGVD 1, AGVD2, AGVD3 and AGVD 17 represent four examples of S.

tuberosum having various levels of homozygosity." *Id.* Applicant also argues that Applicant has demonstrated "the methods that can be used to produce (1) a diploid potato breeding line, (2) hybrid potato seed and (3) an off spring of a diploid potato breeding line of the species of *Solanum tuberosum*." *Id.*

Response to Applicant's Arguments

The rejection is maintained for the reasons stated above. Applicant's use of the word "as" still encompasses a large genus.

15. Claims 1-19 are rejected under the written description requirement because claims 1 and 5 make reference to database accession numbers in the claims: EST724115 and EST717227. It is the Office's interpretation that it is Applicant's clear intent to incorporate the essential material cited by reference to the accession numbers into the claims. The meaning and scope of the claim limitations can only be understood by reference to the accession numbers, however, such an incorporation is improper.

The incorporation of essential material in the claims by reference to an unpublished U.S. application, foreign application or patent, to a publication, or to an online database is improper. Applicant is required to amend the claims to include the material incorporated by reference, if the material is relied upon to overcome any objection, rejection, or other requirement imposed by the Office. The amendment must be accompanied by a statement executed by the applicant, or a practitioner representing the applicant, stating that the material being inserted is the material

previously incorporated by reference and that the amendment contains no new matter. 37 CFR 1.57(f).

Dependent claims are included in this rejection because none provide further limitations obviating this rejection.

Applicant's Arguments and Response

Due to Applicant's amendments to the claims, the rejection is modified from the rejection set forth in the Office action mailed 14 March 2016 as applied to the above-referenced claims. Applicant traverses the rejection in the paper filed 14 September 2016. Applicant's arguments have been fully considered but are not persuasive. Applicant states that the specification was amended to incorporate the material, however, no such amendment was made. In any case, such an amendment would not be allowed under 37 CFR 1.57. The incorporation of essential material into specification by reference to an unpublished U.S. application, foreign application or patent, to a publication, or to an online database is improper.

16. Claims 1-19 are rejected under 35 U.S.C. 112(a) or 35 U.S.C. 112 (pre-AIA), first paragraph, as failing to comply with the enablement requirement. The claim(s) contains subject matter which was not described in the specification in such a way as to enable one skilled in the art to which it pertains, or with which it is most nearly connected, to make and/or use the invention.

Due to Applicant's amendments to the claims, the rejection is modified from the rejection set forth in the Office action mailed 14 March 2016 as applied to the above-referenced claims. Applicant traverses the rejection in the paper filed 14 September 2016. Applicant's arguments have been fully considered but are not persuasive.

The invention appears to employ novel plants. Since the plant is essential to the claimed invention it must be obtainable by a repeatable method set forth in the specification or otherwise be readily available to the public. If the plant is not so obtainable or available, the requirements of 35 USC § 112 may be satisfied by a deposit of the seeds. A deposit of 2500 seeds of each of the claimed embodiments is considered sufficient to ensure public availability.

The specification does not disclose a repeatable process to obtain the plant and thus it is not apparent if the plant is readily available to the public.

(a) If a deposit is made under the terms of the Budapest Treaty, then a statement, affidavit or declaration by Applicant, or a statement by an attorney of record over his or her signature and registration number, or someone empowered to make such a statement, stating that the instant invention will be irrevocably and without restriction released to the public upon the issuance of a patent, would satisfy the deposit requirement made herein.

(b) If a deposit has not been made under the Budapest Treaty, then in order to certify that the deposit meets the criteria set forth in 37 CFR 1.801-1.809 and MPEP 2402-2411.05, Applicant may provide assurance of compliance by statement, affidavit

or declaration, or by someone empowered to make the same, or by a statement by an attorney of record over his or her signature and registration number showing that:

(i) during the pendency of this application, access to the invention will be afforded to the Commissioner upon request;

(ii) all restrictions upon availability to the public will be irrevocably removed upon granting of the patent in accordance with 37 CFR § 1.808(a)(2);

(iii) the deposit will be maintained in a public depository for a period of 30 years or 5 years after the last request or for the effective life of the patent, whichever is longer;

(iv) a test of the viability of the biological material at the time of deposit (see 37 CFR § 1.807); and,

(v) the deposit will be replaced if it should ever become inviable.

In addition, the identifying information set forth in 37 CPR 1.809(d) should be added to the specification, if not already present. See 37 CPR 1.801 - 1.809 (MPEP 2401-2411.05) for additional explanation of these requirements.

Applicant has deposited seeds (see e.g. pages 34-35 of the instant specification). The deposit was made under the terms of the Budapest treaty. Response, p. 15. Examiner also notes that, inter alia, a statement, affidavit or declaration by Applicant, or a statement by an attorney of record over his or her signature and registration number, or someone empowered to make such a statement, stating that the instant invention will

be irrevocably and without restriction released to the public upon the issuance of a patent is required.

Applicant's Arguments and Response

Applicant states that the deposit was made under the terms of the Budapest treaty, obviating part of the rejection. Applicant, however, has not affirmed that the instant invention will be irrevocably and without restriction released to the public upon the issuance of a patent. This statement is required.

17. Claims 1-5 and 7-19 are rejected under 35 U.S.C. 112(a) or 35 U.S.C. 112 (pre-AIA), first paragraph, because the specification, while being enabling for the deposited cell lines, does not reasonably provide enablement for forms of self-compatibility as broadly as claimed. The specification does not enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the invention commensurate in scope with these claims.

This is a new rejection necessitated by Applicant's amendments to the claims.

The invention appears to employ plants with novel characteristics. Since these plant characteristics are essential to the claimed invention it must be obtainable by a repeatable method set forth in the specification or otherwise be readily available to the public. If the plant is not so obtainable or available, the requirements of 35 USC § 112 may be satisfied by a deposit of the seeds. Deposits under NCIMB accession numbers

41663, 41664, 41665 and 41765 have been made. However the claims are not drawn solely to the deposits.

Claim 1 is drawn to a diploid, fertile, self-compatible and essentially homozygous potato line having an average tuber yield expressed in grams of fresh weight of at least 200 grams per plant; wherein said line additionally comprises 1-6 agronomically desirable traits or genes selected from a Markush group and wherein the self-compatibility originates from *S. chacoense* and is as present in a plant of potato lines NCIMB accession number 41663, NCIMB accession number 41664, NCIMB accession number 41665, or NCIMB accession number 41765, further where representative seeds were deposited with the NCIMB under breeders reference AGVD1, AGVD2, AGVD3, and AGVD17.

Claims depending from claim 1 read on a hybrid potato seed produced from a cross between two essentially homozygous parental breeding lines and a potato plant(s) produced therefrom. Claim 8 reads on a method using a plant from claim 1. Claims 9-13 read on plants and plant parts produced by the method of claim 8. Claim 14 reads on a tissue culture produced by the plant of claim 10 and claim 15 reads on a plant produced from the tissue culture of claim 14. Claim 16 reads on a method using a seed produced according to claim 8 to produce a plant; claim 17 reads on a plant or tuber produced according to claim 16. Claim 18 reads on a set of at least 2 intercrossable diploid, fertile, self-compatible and essentially homozygous potato lines comprising plants according to claim 1.

Claim 5 is a method claim drawn to producing a diploid, fertile, self-compatible and essentially homozygous potato line starting with a first potato plant of a first diploid self-compatible, fertile and essentially homozygous potato line wherein the self-compatibility originates from *S. chacoense* and is as present in a plant of potato lines NCIMB accession number 41663, NCIMB accession number 41664, NCIMB accession number 41665, or NCIMB accession number 41765, further where representative seeds were deposited with the NCIMB under breeders reference AGVD1, AGVD2, AGVD3, and AGVD17 and crossing it to a second potato plant where the second potato plant has an average tuber yield expressed in grams of fresh weight of at least 200 grams per plant and then selfing or backcrossing to provide a diploid, self-compatible and essentially homozygous potato breeding line comprising plants having an average tuber yield of at least 200 grams per plant, and then introducing into a plant of said line at least one trait or gene selected from a Markush group. Claim 6 recites specific deposited cell lines; claim 7 encompasses a diploid potato breeding line obtained by the method of claim 5. Claim 19 reads on an elite diploid potato breeding line obtained by the method of claim 5.

The claimed genera, in particular, include those set forth in the following exemplary claims:

claim 1 – any “diploid, fertile, self-compatible and essentially homozygous potato line” wherein the self-compatibility originates from *S. chacoense* and is as present in a plant of potato lines NCIMB accession number 41663, NCIMB accession number 41664,

NCIMB accession number 41665, or NCIMB accession number 41765 together with at least one agronomically desirable trait;

claim 5 – any “diploid, fertile, self-compatible and essentially homozygous potato line” wherein the self-compatibility originates from *S. chacoense* and is as present in a plant of potato lines NCIMB accession number 41663, NCIMB accession number 41664, NCIMB accession number 41665, or NCIMB accession number 41765 as a starting material in part (a) to produce a “diploid, fertile, self-compatible and essentially homozygous potato line” with an added trait or gene.

The above claims, however, are not limited to the self-compatibility gene and potato lines described by the deposited accessions. The claims encompass the genus of all self-compatibility genes that are “as present” in the deposited lines. Applicant fails to describe the scope of “as present.” For example, the meaning of "as" includes "for instance" and thus the claims also read on other embodiments of self-compatibility genes and potato lines. Merriam-Webster, "as", accessed 2 December 2016.

The Federal Circuit in *In re Wands* lists eight considerations for determining whether or not undue experimentation would be necessary to practice an invention. *In re Wands*, 858 F2d 731, 8 USPQ2d 1400, 1406 (Fed. Cir. 1988). These factors are: the quantity of experimentation necessary, the amount of direction or guidance presented, the presence or absence of working examples of the invention, the nature of the invention, the state of the prior art, the relative skill of those in the art, the predictability or unpredictability of the art, and the breadth of the claims. *Id.*

In the specification, Applicant teaches obtaining the IVP07-1001/4 line from publically available germplasm and plants that contain the *Sli* gene and using these plants to breed AGVD1, AGVD2, and AGVD3. Specification ("Spec."), pp. 46-47. Applicant teaches that lines were deposited and that the deposited lines are known by their breeders reference: AGVD1 (NCIMB 41663), AGVD2 (NCIMB 41664), AGVD3 (NCIMB 41665) and AGVD17 (NCIMB 41765). Id., pp. 34-35. Applicant teaches a breeding process according to the instant invention starting with AGVD1-3 or AGVD17 on page 39 et seq.

Applicant teaches crossing the *Sli* gene donor with various potato clones designated IVP97-079-9, IVPAA-096-I8 and SH83-92-488 to generate clones D1, D2 and D3. Id., p. 49. Applicant teaches creating a line containing the *Sli* gene with enhanced agricultural quality in Example 2, pp. 48-51. Applicant teaches creating a molecular marker platform, i.e., SNPs, in Example 3, p. 51 et seq. In Example 4, Applicant teaches the development of homozygous diploid potato plants and then F1 hybrid progeny, as well as subsequent generations. Id., p. 60 et seq. Applicant teaches the meaning of the claim term "essentially homozygous" on pages 21-22 of the instant specification and suggests that the term reflects a level of homozygosity that is above 25% or 75%. Id., p. 21-22. Applicant generally teaches adding an agronomically desirable trait to a potato genotype on, e.g. page 37.

The art generally teaches this breeding strategy, or similar strategies. For example, Jansky teaches first obtaining homozygous diploids using a self-incompatibility inhibitor. J Jansky, "Breeding, Genetics, and Cultivar Development,"

Advances in Potato Chemistry and Technology, ed. J. Singh & L. Kaur, pp. 27-62 (June, 2009), p. 46. Jansky teaches that "[h]ighly homozygous inbreds could be also be used for heterosis breeding. Jansky, p. 46. Jansky teaches that such an approach would be difficult. *Id.*

Phumichai et al. confirms the approach of Jansky, and documents the use of the *Sli* gene in the approach. Phumichai et al., Genome 48:977-84 (2005), abstract.

> Potato (Solanum tuberosum) is an auto- [sic] or segmental allo-tetraploid showing tetrasomic inheritance, which results in complicated genetic segregation. Since potatoes are propagated vegetatively, highly heterotic genotypes can be maintained, which facilitates the use of heterosis, an essential factor in potato breeding, particularly for tuber yield. Alternatively, open-pollinated true potato seed (TPS) can be used as a low-cost planting material for potato production.
>
> Highly homozygous inbreds, once amply produced, would be highly useful for the development of TPS varieties with genetic uniformity, as well as for the investigation of heterosis."
>
> "A scheme for hybrid breeding using homozygous diploids and subsequent somatic hybridization has been proposed, for which the availability of highly homozygous diploid (or pure) lines is a key for success. In considering the use of pure lines for the development of genetically uniform [true potato seed] varieties, self-fertile, seed-propagated lines are highly desirable, and simple techniques to develop such lines are necessary." .

Id., pp. 977-78 (citations omitted).

In the response filed on 14 September 2016, Applicant traverses the rejection under 35 USC 103 and argues that one of skill in the art would not have a reasonable expectation of success. Response, pp. 16-22. As part of the argument, Applicant reviews the scope and content of the prior art, including the references cited above. For example, Applicant cites to a publication from Watanabe et al. (Breed Sci 46:329-36

(1996)) as standing for “homozygous inbred lines have not been obtained at the diploid level due to both inbreeding depression and self-incompatibility.” Response, p. 17. Applicant also cites to Jansky as downplaying the possibility of hybrid breeding as ‘commercially unfeasible due to "dramatic reductions in fertility and vigor.'" Response, p. 17 (quoting Jansky, p. 46). Applicant also argues that Uijtewaal et al. (Euphytica, 36: 745-753, 1987) confirms this teaching. Response, p. 17 & p. 19. Applicant concludes the argument with citation of additional material supporting their position that one of skill in the art would not have a reasonable expectation of success in combining the art cited in the rejection under 35 USC 103.

In support of the argument, Applicant also filed a declaration under 37 CFR 1.132 by Dr. Lindhout with supporting letters. Dr. Lindhout’s declaration was formally filed in application serial no. 13/504,156, and the supporting letters were initially filed in European Application EP2699684 (04 June 2014) and are copies therefrom. The letters teach that the success of Applicant's invention, as broadly as claimed, was unpredictable. For example, on page 2 of his letter, Dr. Koornneef states that

> Ever since Lindhout and his co-workers published their results in Potato Research volume 54 of 2011, showing high yielding hybrids from 87% homozygous F3 inbreds, I have been positively surprised that Solynta has managed to challenge this dogma. Solynta could successfully obtain essentially homozygous inbred lines by making use of genotypes that are selfcompatible and by choosing the right starting material that apparently is heterozygous for a lower number of deleterious alleles. This provides the basis of generating useful inbred lines that can become parents of F1 hybrids.

Letter from Koornneef (2012) p. 2 (underlining added). Earlier, Dr. Koornneef also states that “to start a project aiming at developing a good performing homozygous

potato plant for the purpose of hybrid production is a daring undertaking with very little chance of success. *Id.*

In another letter supplied in support of Dr. Lindhout's declaration, Dr. Jones states that

> "[I]n the mid 2000s we attempted to produce hybrid potato ourselves. . . . However, we never succeeded in developing any promising material in the form of fertile homozygotes that could be useful for hybrid breeding and that could tolerate recurrent selfing. . . . We considered that this was due to our inability to cross the Sli- self-compatibility locus into good enough or diverse enough diploid *Solanum* germplasm.

Letter from Dr. Jonathan D.G. Jones (2012), p. 1 (underlining added).

Applicant claims a large genus of potato plants. Jansky and Spooner et al. describes the breadth of the of the claimed genus as extremely large. See, e.g., Jansky, "Breeding, Genetics, and Cultivar Development," Advances in Potato Chemistry and Technology, ed. J. Singh & L. Kaur, pp. 27-62 (June, 2009), pp. 27-29; Spooner et al., Bot Rev 80:283-383 (2014), Table 1, pp. 286-94. Applicant's claimed invention reads on any potential potato line that is a "diploid, fertile, self-compatible and essentially homozygous potato line" where the self-compatibility is "as present" in the deposited samples.

In view of Applicant's arguments in response to the rejection under 35 USC 103 and Dr. Lindhout's supporting letters, success in practicing Applicant's claimed invention would be unpredictable and would depend on the genotype of the potato lines. Therefore, extensive guidance would be required to enable the invention as broadly as claimed. Such guidance is not provided.

The specification therefore does not enable one skilled in the art to make and use members of the species of the claimed genus other than the deposited lines. See, for example, MPEP 2164.06(a)(II), citing to *Ex parte Jackson*, 217 USPQ 804, 806 (Bd. App. 1982). The issue before the Board in *Ex parte Jackson* was:

> The issue squarely raised by this rejection is whether or not a description of several newly discovered strains of bacteria having one particularly desirable metabolic property in terms of the conventionally measured culture characteristics and a number of metabolic and physiological properties would enable one of ordinary skill in the relevant art to independently discover additional strains having the same specific, desirable metabolic property, i.e., the production of a particular antibiotic.
>
> To state the issue somewhat differently, it is whether a verbal description of a new species would enable one of ordinary skill in the relevant art to obtain strains of that species over and above the specific strains made available through deposit in one of the recognized culture depositories.

Ex parte Jackson, 217 USPQ 804, 806 (Bd. App. 1982). In the instant application, the issue is similar to the one before the Board in that Applicant made deposits with unique characteristics but not taught how to identify other lines. In *Ex parte Jackson*, the Board held that the deposit, without a detailed explanation of how to obtain more species from nature, did not enable the generic claims. *Id.*, p. 808. The board found that "the degree of experimentation involved in locating new [organisms] apart from deposited cultures is undue in light of the enablement requirement of 35 U.S.C. 112." *Id.* The *Jackson* decision was considered and distinguished by the Federal Circuit in the *Wands* decision in view of the routine experimentation required to obtain hybridomas. *In re Wands*, pp. 1403-04. Therefore, in view of the *Ex parte Jackson* decision, the generic scope of claims 1 and 5, reading on the genus of as set forth above is not enabled. Obtaining

further species falling within the scope of the generic claim would require undue experimentation.

Dependent claims are included in this rejection because none provide further limitations obviating this rejection.

Double Patenting

The nonstatutory double patenting rejection is based on a judicially created doctrine grounded in public policy (a policy reflected in the statute) so as to prevent the unjustified or improper timewise extension of the "right to exclude" granted by a patent and to prevent possible harassment by multiple assignees. A nonstatutory obviousness-type double patenting rejection is appropriate where the conflicting claims are not identical, but at least one examined application claim is not patentably distinct from the reference claim(s) because the examined application claim is either anticipated by, or would have been obvious over, the reference claim(s). See, e.g., *In re Berg*, 140 F.3d 1428, 46 USPQ2d 1226 (Fed. Cir. 1998); *In re Goodman*, 11 F.3d 1046, 29 USPQ2d 2010 (Fed. Cir. 1993); *In re Longi*, 759 F.2d 887, 225 USPQ 645 (Fed. Cir. 1985); *In re Van Ornum*, 686 F.2d 937, 214 USPQ 761 (CCPA 1982); *In re Vogel*, 422 F.2d 438, 164 USPQ 619 (CCPA 1970); and *In re Thorington*, 418 F.2d 528, 163 USPQ 644 (CCPA 1969).

A timely filed terminal disclaimer in compliance with 37 CFR 1.321(c) or 1.321(d) may be used to overcome an actual or provisional rejection based on a nonstatutory double patenting ground provided the conflicting application or patent either is shown to be commonly owned with this application, or claims an invention made as a result of activities undertaken within the scope of a joint research agreement.

Effective January 1, 1994, a registered attorney or agent of record may sign a terminal disclaimer. A terminal disclaimer signed by the assignee must fully comply with 37 CFR 3.73(b).

The USPTO internet Web site contains terminal disclaimer forms which may be used. Please visit http://www.uspto.gov/forms/. The filing date of the application will determine what form should be used. A web-based eTerminal Disclaimer may be filled out completely online using web-screens. An eTerminal Disclaimer that meets all requirements is auto-processed and approved immediately upon submission. For more

information about eTerminal Disclaimers, refer to
http://www.uspto.gov/patents/process/file/efs/guidance/eTD-info-I.jsp.

18. Claims 1-19 are provisionally rejected on the ground of nonstatutory obviousness-type double patenting as being unpatentable over claims 3 and 9 of copending Application No. 13/504,156. Although the conflicting claims are not identical, they are not patentably distinct from each other. This is a provisional double patenting rejection since the conflicting claims have not yet been patented.

Claims 3 and 9 of the 13/504,156 application relate to potato lines/plants of a "diploid, fertile, self-compatible and essentially homozygous potato line" comprising agronomically desirable traits and methods using such lines to create plants comprising agronomically desirable traits. The specifications of the two applications teach the same deposited potato lines. Claims 3 and 9 of the 13/504,156 application claim a broad genus, including, e.g., pest/disease resistance, while the instant claim set encompass a sub-genus of the claimed genus listing numerous specific examples of genes associated with, e.g., pest/disease resistance. Claims 1 and 5 generally recite various embodiments of disease resistance, e.g. claim 1, lines 9-17, using terms that are currently rejected under 35 USC 112(b). Therefore the instant application reads on a sub-genus of the genus claimed in the 13/504,156 application.

Applicant's Arguments

Applicant traverses the rejection on pages 28-29 of the response. Applicant argues that the rejection should be made only when the claims of the rejected application are obvious variants of the previous application. *Id.*, p. 29.

Response to Applicant's Arguments

Claim 3 of the '156 application reads generically on adding a disease resistance gene while instant claim 1 reads on various generic embodiments of pest/disease resistance the exact identity of which is not defined. The claim terms are not defined because, in part, of the rejection under 35 USC 112(b).

Conclusion

19. No claim is allowed.

20. The prior art made of record and not relied upon is considered pertinent to Applicant's patent application.
Trofimov et al., Kartofel' i Ovoshchi 6 (2007) teaches developing diploid inbreds in, e.g. pages 4-5 of the translation. The translation only became available in May 2016, after the mailing of the Office action of 14 March 2016.

Applicant's amendment necessitated the new ground(s) of rejection presented in this Office action. Accordingly, **THIS ACTION IS MADE FINAL**. See MPEP § 706.07(a). Applicant is reminded of the extension of time policy as set forth in 37 CFR 1.136(a).

A shortened statutory period for reply to this final action is set to expire THREE MONTHS from the mailing date of this action. In the event a first reply is filed within TWO MONTHS of the mailing date of this final action and the advisory action is not mailed until after the end of the THREE-MONTH shortened statutory period, then the shortened statutory period will expire on the date the advisory action is mailed, and any extension fee pursuant to 37 CFR 1.136(a) will be calculated from the mailing date of the advisory action. In no event, however, will the statutory period for reply expire later than SIX MONTHS from the date of this final action.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to RUSSELL BOGGS whose telephone number is (571)272-2805. The examiner can normally be reached on Monday through Friday between 8:00 AM to 6:30 PM (Mountain Time).

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Amjad Abraham, can be reached at 571-270-7058. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov. Should you have questions on access to the Private PAIR system, contact the Electronic

Business Center (EBC) at 866-217-9197 (toll-free). If you would like assistance from a USPTO Customer Service Representative or access to the automated information system, call 800-786-9199 (IN USA OR CANADA) or 571-272-1000.

/RUSSELL BOGGS/
Examiner, Art Unit 1663

/AMJAD ABRAHAM/
Supervisory Patent Examiner, Art Unit 1663

I hereby certify that this paper (along with any paper referred to as being attached or enclosed) is being transmitted via the Office electronic filing system in accordance with 37 CFR § 1.6(a)(4).

Dated: 2/15/17 Signature: (Michelle DiFalco)

Docket No.: 091876-0027 **PATENT**

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of: Willem Hendrik LINDHOUT, et al.	:	Customer Number: 23630
Application No.: 14/112,747	:	Confirmation No.: 5919
Filed: December 23, 2013	:	Art Unit: 1663
For: Hybrid Seed Potato Breeding	:	Examiner: Russell T. BOGGS

AMENDMENT IN RESPONSE TO FINAL OFFICE ACTION

Mail Stop - Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

INTRODUCTORY COMMENTS

In response to the Final Office Action dated December 15, 2016, please amend the above-identified U.S. patent application as directed herein. A Petition for Extension of Time (3 months), an Information Disclosure Statement and a Request for Continued Examination (RCE) are concurrently filed herewith. Reconsideration and further examination of the present application is requested in light of the following

Amendments to the Specification begin on Page 2.

Amendments to the Claims begin on Page 11.

Remarks/Arguments begin on Page 22.

AMENDMENTS TO THE SPECIFICATION

In the following amendment to the specification, ~~deletions are shown with strike through~~.

Please amend the paragraph at page 53, lines 17-22 as follows:

The 100 SNP sequences selected as described above were used in a KASPar SNP genotyping system (KBioscience Ltd. Hoddesdon, UK; ~~http://~~www.kbioscience.co.uk/lab%20services/SNP%20Genotyping/genotyping_chemistry.html). The markers were tested on a set of 20 diploid potato genotypes (see Figure 4) including the three diploid parents used in the present study and further on the donor of the *Sli* gene, clone 07-1004-1 (see also Example 2 herein above).

Please amend Table 10 at pages 84 to 90 as follows:

	class	trait name	locus symbol	*germplasm source*	marker/ Chromosomal position	encoded protein	GenBank acession number	literature references
1								
2	Tuber morphological traits							
3	D	Red anthocyanin	*D (R/Rpw)*	*Solanum tuberosum L.*	Chrom. 3	dihydroflavonol 4-reductase (DFR)	AY288924.1	Van Eck et al., (1994); De Jong et al., (2003)
4	D	Blue or purple pigments	*P*	*Solanum tuberosum L.*	Chrom. 11	flavonoid 3',5'-hydroxylase (F3'5'H)	AY675561 recessive allele cv. Rideau AY675560 recessive allele clone USW5281.2 AY675558 recessive allele clone USW5281.2 AY675559 dominant allele Jung et al clone USW5281.2	Jung et al., (2005)

5	D	Pigmentation of tuber skin	*I, (D* in tetraploids)	*Solanum tuberosum L.* Cultivar WS281.2	Chrom. 10	Stan2 is an R2R3 MYB transcription factor anthocyanin 1 (an1)	A Stan2 (777) cDNA isolated from WS281.2 (AY841129)	De Jong, (1987); Jung et al., (2009)
6	C	Pigmented tuber flesh colour	*Pf*	*Solanum tuberosum L.*	Chrom. 10	an allelic variant or paralog of *Stan2* R2R3 MYB transcription factor		De Jong, (1987); Zhan et al., (2009)
7	C	Yellow flesh colour	*Y-locus or BCH or Chy2*	*Solanum tuberosum L.*	Chrom. 3	beta-carotene hydroxylase 2	HM013963	Bonierbale et al., (1988); Brown et al., (2006)
8	C	Orange flesh colour	*Chy2 + zep* ZEP-1 allele ZEP-2 allele	*Solanum tuberosum L.*	Chrom. 2	*Zep* (on Chromosome 2) is involved in the conversion of zeaxanthin into antheraxanthin, and in the conversion of antheraxanthin into violaxanthin	HM013964 and HM013965	Wolters et al, (2010)
9	B	Tuber shape	*Ro*	*Solanum tuberosum L. CxE* US W5337.3	Chrom. 10, TG34 - TG63			Van Eck et al., 1994. Genetics 137: 303-309
10	Processing or tuber quality traits							
11	D	Amylose / amylopectine	*GBSS*	*Solanum tuberosum L.*	Chrom. 8	granule-bound starch synthase	X58453.1	Van der Leij et al., (1991)
12	C	chipping quality cold sweetening	PAIN-1	*Solanum tuberosum L.*	Chrom. 3	beta-fructofuranosidase or potato acid invertase	X70368.1	Li et al., (2008).
13	D	Starch content and french fry quality	SPS	*S.tuberosum*	Chrom. 7	sucrose phosphate synthase (sps)	EU684230.1, X73477.1	Zrenner et al., (1995)

14	C	chipping quality cold sweetening	InvGE/GF	*Solanum tuberosum L.*	Chrom. 9	acid invertase invertase apoplastic invertase (InvCD141) invertase (InvCD111) gene	AY341425.1 AJ133765, HQ110081, HQ197977	Li et al., (2008).
15	D	chipping quality cold sweetening	invertase inhibitor	*Solanum tuberosum L.*	Chrom. 12	Isoforms of an invertase inhibitor	AY864819 INH1*A allele AY864820 INH1*B allele FJ810205 INH2alpha*A1 allele FJ810206 INH2alpha*A2 allele FJ810207 INH2alpha*B allele FJ810208 INH2alpha*C allele FJ810209 INH2alpha*D allele AY864821 INH2beta*A1 allele GU980593, GU980594 INH2b*B2 Cryptic? 158 1 Vacuolar GU980595	Brummel et al., (2011).
16	D	Black spot bruising	*PPO*	*Solanum tuberosum L.*	Chrom. 8	Polyphenoloxidase PPO	POT33 allele U22922.1POT32 allele U22921.1POT72 allele U22923.1	Thygesen et al., (1995)
17	D	Starch content and french fry quality	Sucrose synthase	*Solanum tuberosum.*	Chrom. 9	sucrose synthase SuSy2	AY205302	
18	D	phenolic antioxidants	*StMtf1M*	*Solanum tuberosum L. chlorogenate inducer (Cai1) gene*		MYB transcription factor gene StMtf1M to activate the phenylpropanoid biosynthetic pathway	EU310399	Rommens et al., (2008a)
19	D	acrylamide levels	*StAs1 and StAs2*	*Solanum tuberosum L.*		asparagine synthetase	CK278037.1 (EST734115) CK271149.1 (EST717227)	Rommens et al., (2008b)

20	D	Starch phosphate content and frying quality	*PhL*	*Solanum tuberosum L.*		Starch phosphorylase-L (PhL)	AF143202	Rommens et al., (2006)
21	D	Starch phosphate content and frying quality	*GWD*	*Solanum tuberosum L.*	Chrom. 5	R1 protein or synonymous with Glucan Water Dikinase	YD9533.1	Ritte et al., (2002); Rommens et al., (2006)
22	D	Resistance to high temperature inhibition of tuber formation	*FT*	*Solanum tuberosum L.*		Flowering locus T protein	GU223211.1	Fan et al., (2010)
23	Resistance to *Phytopthera infestans*							
24	D	Resistance to *Phytophthera infestans*	*R1*	*Solanum demissum*	Chrom. 5	R1	AF447489.1	Leonards-Schippers *et al.* (1994); Ballvora, et al., (2002)
25	D	Resistance to *Phytophthera infestans*	*R2*	*Solanum demissum*	Chrom. 4	Late blight resistance gene R2	FJ536325.1	Lokossou et al., (2009)
26	D	Resistance to *Phytophthera infestans*	*R2-like*	*Solanum sp. AM-3778-16*	Chrom. 4	AM-3778-16 R2-like protein gene	FJ536323.1	Lokossou et al., (2009)
27	D	Resistance to *Phytophthera infestans*	*Rpi-hjt1.3, Rpi-hjt1.2, Rpi-hjt1.1*	*Solanum hjertingii*	Chrom. 4	Rpi-hjt1.3, Rpi-hjt1.2, Rpi-hjt1.1	Rpi-hjt1.3 GU563973.1 Rpi-hjt1.2 GU563972.1 Rpi-hjt1.1 GU563971.1	Champouret, N. (2010)
28	D	Resistance to *Phytophthera infestans*	*Rpi-abpt*	*Solanum bulbocastanum*	Chrom. 4	ABPT Late Blight resistance gene Rpi	FJ536324.1	Lokossou et al., (2009)
29	D	Resistance to *Phytophthera infestans*	*R3a*	*Solanum demissum*	Chrom. 11	R3a	AY849382	El-Kharbotly et al. (1994); Huang et al., (2004; 2005)
30	D	Resistance to *Phytophthera infestans*	*R3b*	*Solanum demissum*	Chrom. 11	R3b	HQ437670.1	El-Kharbotly et al. (1994); Huang et al., (2004)

31	B	Resistance to *Phytophthera infestans*	*Rpi1*	*Solanum pinnatisectum* PI 253214	Chrom. 7	Rpi1		Kuhl et al. (2001)
32	D	Resistance to *Phytophthera infestans*	*Rpi-vnt1.1*	*Solanum venturii*	Chrom. 9	Late blight resistance protein Rpi-vnt1.1 gene	FJ423044.1	Foster et al., (2009); Pel et al., (2009)
33	D	Resistance to *Phytophthera infestans*	*Rpi-vnt1.2*	*Solanum venturii*	Chrom. 9	Late blight resistance protein Rpi-vnt1.2 gene	FJ423045.1	Foster et al., (2009)
34	D	Resistance to *Phytophthera infestans*	*Rpi-vnt1.3*	*Solanum venturii*	Chrom. 9	Late blight resistance protein Rpi-vnt1.3 gene	FJ423046.1	Foster et al., (2009)
35	D	Resistance to *Phytophthera infestans*	*Rpi-blb1 / Rb*	*Solanum bulbocastanum* *BGRC accession 8005* Clone PT29	Chrom. 8 BAC clone CB3A14	putative disease resistant protein RGA2 gene Blight resistance protein RPI gene Rpi-blb1 / Rb	AY336128 / AY426259 / AY303170	Van der Vossen et al., (2003); Song et al., (2003)

36	D	Resistance to *Phytophthera infestans*	*Rpi-blb2*	*Solanum bulbocastanum* *Clone ABD 1197-16;* *Clone 6B99-256-3*	Chrom. 6	Rpi-blb2 Rpi-blb3	DQ122125.1	Lokossou et al., (2009)
37	D	Resistance to *Phytophthera infestans*	*Rpi-bt1*	*Solanum bulbocastanum* *Somatic hybrid J101*	Chrom. 8, between CD60 and TG261	Rpi-bt1	FJ188415.1	Oosumi et al., (2009)
38	B	Resistance to *Phytophthera infestans*	*R5*	*Solanum demissum* *Black 3053-18*	Chrom. 11	R5		Huang S (2005)
39	B	Resistance to *Phytophthera infestans*	*R6*	*Solanum demissum* *Tetraploid clone Mal86*	Chrom. 11	R6		El-Kharbotly et al. (1996)
40	B	Resistance to *Phytophthera infestans*	*R7*	*Solanum demissum* *Tetraploid clone Mal87*	Chrom. 11	R7		El-Kharbotly et al. (1996)
41	A	Resistance to *Phytophthera infestans*	*R8*	*Solanum demissum* *Host 2424o(5)*		R8		Black et al., (1953); Malcolmson et al., (1966).
42	B	Resistance to *Phytophthera infestans*	*R9*	*Solanum demissum* *Host 2573(2)*	Chrom. 11	R9		Malcolmson et al., (1966), Huang S (2005)
43	B	Resistance to *Phytophthera infestans*	*R10*	*Solanum demissum* *Black 3681 ad (1)*	Chrom. 11	R10		Huang S (2005)
44	B	Resistance to *Phytophthera infestans*	*R11*	*Solanum demissum* *Black 5008 ab (6)*	Chrom. 11	R11		Huang S (2005)

45	8	Resistance to *Phytophthera infestans*	*Rpi-ber1*	*Solanum berthaultii Hybrid of S. tuberosum dihaploid USW2230 x Solanum berthaultii PI473331*	Chrom. 10, between CT214 and TG63	Rpi-ber1		Ewing et al. 2000; Rauscher et al., (2006) ; Park et al., (2009)
46	8	Resistance to *Phytophthera infestans*	*Rpi-mcd1*	*Solanum microdontum BGRC 18302 (CGN21342)*	Chrom. 4			Tan et ., (2008); Tan et al., 2010)
47	Resistance to nematodes							
48	8	Resistance to *G. rostochiensis Ro1*	*H1*	*Solanum tuberosum subsp. andigena CPC ~~1630 (or 1573)[?]~~*	Chrom. 5, linked to CP113 and CD78			*Pineda et al.* (1993); Gebhardt *et al.* (1993)
49	8	Resistance to *G. pallida*	*H3 or $Gpa1V_{vrn}$*	*Solanum tuberosum subsp. Andigena CPC accession 2802*	Chrom. 4; Marker assay:8AC9_3_fwd_ CGCCAACTCTGCTTC ATACABAC9_3_rev_ TGCAGAGGTCAGTTG TTTGG. SNP markers were developed from BACs GB003E01 and GB080G22 submitted to GenBank as AC236732 and AC236733, respectively.			Bradshaw et al., (1998); Moloney et al., (2010)

50	D	Resistance to *G. rostochiensis* Ro1	*Gro1-4*	*Solanum spegazzinii*	Chrom. 7		AY196151	Paal, et al., (2004)
51	B	Resistance to *Globodera rostochiensis* Ro5-22 and *G. pallida* Pa2-D383	*Grp1*	*Solanum tuberosum* 78-3778 *(AM)*	Chrom. 5			Rouppe van der Voort et al., (1998)
52	B	Resistance to *G. pallida* Pa2, Pa3	*Gpa*	*Solanum spegazzinii* BGRC accession 6218 seedling number 15	Chrom. 5			Kreike *et al.* 1994
53	C	Resistance to *G. pallida*	*Gpa2*	*Solanum tuberosum Subsp. Andigena Cpc 1673*	Chrom. 12, between GP34 and CT79		AF195939.1	Van der Vossen et al., (2000); Bakker et al., (2003).
54	B	Resistance to *G. pallida* Pa2, Pa3	*GpaX1 $_{tbr}$ 1*	*Solanum torijense* BGRC 24717	Chrom. 11			Tan et al., (2009).
55	B	Resistance to *G. pallida* pathotype Pa2, Pa3	*Gpa3*	*3704-76 (JP)*	Chrom. 5: forward primer 5' ACACCACCTGTTTGA TAAAAAACT 3' with the reverse primer 5' GCCTTACTTCCCTGCT GAAG 3' resulted in haplotype c			Rouppe van der Voort *et al.* (2000); Sattarzadeh et al., (2006)
56	B	Resistance to *G. pallida* pathotype Pa3	*Gpa6*	*3704-76*	Chrom. 9			Rouppe van der Voort et al. (2000); Sattarzadeh et al., (2006)
57	B	Resistance to *M. chitwoodi*	R_{mc1}	*Solanum bulbocastanum* 275187.1	Chrom. 11			Brown *et al.* (1996); Rouppe van der Voort et al. (1999)
58	B	Resistance to *M. chitwoodi/M. fallax*	*RMc2*	*Solanum fendlerii* BGRC 8083	Chrom. 11			Grassistra (2006)

59	B	Resistance to *M. chitwoodi/M. fallax*	*RMc1(hou)*	*Solanum hougasii* PI 161726	Chrom. 11			Brown *et al.*, (2003)
60	A	Resistance to *M. hapla*	*RMh1*	*Solanum chacoense* BGRC18618				Draalstra (2006)
61	A	Resistance to *M. hapla*	*RMh2*	*Solanum chacoense* BGRC18618				Draalstra (2006)
62	A	Resistance to *M. hapla*	*RMh3*	*Solanum tarijense* BGRC 24717				Draalstra (2006)
63	Resistance to viruses							
64	C	Resistance to PVY	Ry_{sto}	*Solanum tuberosum subsp andigena*	Chrom. 11		Y15297.1; AJ300266.1 and splice variants C38 and C39	Hämäläinen *et al.* (1997); Sorri et al (1999); Kasai et al., (2000); Vidal et al., (2002)
65	B	Resistance to PVY	*Rysto*	*Solanum stoloniferum*	Chrom. 12			Song, et al., (2005; 2008)
66	D	Resistance to PVX	*Rx*	*Solanum tuberosum subsp andigena CPC 1673**	Chrom. 12		AJ011801.1	Ritter *et al.* (1991); Bendahmane et al. (1999); Kanyuka et al., (1999)
67	B	Resistance to potato virus X (PVX)	*Nb*	*S. tuberosum cv* Pentland Ivory	Chrom. 5, between GP21 and TG432			De Jong *et al.* (1997); Marano et al., (2002)
68	B	Resistance to potato virus S (PVS)	*Ns*	*Solanum tuberosum DW 83-3121*	Chrom. 8, near GP126, GP189 and CP16			Marczewski et *al.*, (2002)

AMENDMENTS TO THE CLAIMS

1. (Currently amended) A diploid, fertile, self-compatible and essentially homozygous *Solanum tuberosum* potato line comprising plants having an average tuber yield expressed in grams of fresh weight of at least 200 grams per plant, wherein said line in addition to said tuber yield comprises at least one, more preferably at least 2, 3, 4, 5 or 6 agronomically desirable traits or genes, wherein said self-compatibility originates from *Solanum chacoense* and is referred to as S-locus inhibitor *(Sli)* gene, as present in a plant of potato lines NCIMB accession number 41663, NCIMB accession number 41664, NCIMB accession number 41665, or NCIMB accession number 41765, representative seeds of said lines having been deposited with the NCIMB, Aberdeen, Scotland under breeders reference AGVD1, AGVD2, AGVD3, and AGVD17 respectively, and wherein said trait or gene is selected from the group consisting of ~~resistance to *Phytophthora infestans* from *Solanum bulbocastanum* or *S. demissum*,~~ *S. bulbocastanum* BGRC 8005-derived *Phytophthora infestans* resistance locus *Rpi-blb1*; *S. bulbocastanum* clone PT29/ BAC clone CB3A14-derived *Phytophthora infestans* resistance gene *Rb* (Genbank acc. No. AY303170); *S. bulbocastanum* clone ARD 1197-16-derived *Phytophthora infestans* resistance locus *Rpi-blb2* (Genbank acc. No. DQ122125); *S. bulbocastanum* clone Blb99-256-3-derived *Phytophthora infestans* resistance locus *Rpi-blb3*; *S. bulbocastanum/S. tuberosum* somatic hybrid J101-derived *Phytophthora infestans* resistance locus Rpi-bt1; *S. demissum* Black 3053-18-derived *Phytophthora infestans* resistance locus R5; *S. demissum* Black tetraploid clone MaR6-derived *Phytophthora infestans* resistance locus R6; *S. demissum* tetraploid clone MaR7-derived *Phytophthora infestans* resistance locus R7; *S. demissum* host 2424 a (5)-derived *Phytophthora infestans* resistance locus R8; *S. demissum* host 2573 (2)-derived *Phytophthora infestans* resistance locus R9; *S. demissum* Black 3681 ad (1)-derived *Phytophthora infestans* resistance locus R10; *S. demissum* Black 5008 ab (6)-derived *Phytophthora infestans* resistance locus R11; ~~resistance to *G. rostochiensis* Ro1 from *S. tuberosum* subsp. *andigena* CPC 1690 or 1673,~~ *S. tuberosum* subsp. *andigena* CPC1673-derived *G. rostochiensis* Ro1 resistance locus H1; ~~resistance to *G. pallida* from *S. tuberosum* subsp. *andigena*,~~ *S. tuberosum* subsp. *andigena* CPC2802-derived *G. pallida* resistance loci *H3* or *GpaIVadg*;

~~resistance to *G. rostochiensis* Ro5-22 and *G. pallida* Pa2-D383 from *S. tuberosum* accession 78-3778,~~ *S. tuberosum* AM78-3778-derived *G. rostochiensis* and *G. pallida* resistance locus *Grp1*; ~~resistance to *G. pallida* Pa2 and Pa3 from *Solanum spegazzinii* and *Solanum tarijense*,~~ *S. spegazzinii* BGRC 8218-15-derived *G. pallida* pathotypes Pa2 and Pa3 resistance locus *Gpa*; *S. tarijense* BGRC 24717-derived *G. pallida* pathotypes Pa2 and Pa3 resistance locus *GpaXI tar 1*; ~~resistance to *G. pallida* Pa3 from *S. tuberosum* accession 3704-76,~~ *S. tuberosum* 3704-76 (JP)-derived *G. pallida* pathotypes Pa2 and Pa3 resistance locus *Gpa5* and pathotype Pa3 resistant locus *Gpa6*; ~~resistance to *M. chitwoodi* from *S. bulbocastanum*,~~ *S. bulbocastanum* PI 275187-derived *M. chitwoodi* resistance locus *Rmc1*; ~~resistance to *M. chitwoodi*/*M. fallax* from *S. fendlerii* and *S. hougassii*,~~ *S. fendlerii* BGRC 8083-derived *M. chitwoodi* and *M. fallax* resistance locus *Rmc2*; *S. hougassii* PI 161726-derived *M. chitwoodi* resistance locus *Rmc1(hou)*; ~~resistance to *M. hapla* from *S. chacoense* and *S. tarijense*,~~ *S. chacoense* BGRC 18618-derived *M. hapla* resistance loci *Rmh1* and *Rmh2*; *S. tarijense* BGRC 24717-derived *M. hapla* resistance locus *Rmh3*; ~~resistance to PVX from *S. tuberosum* cv Pentland Ivory,~~ *S. tuberosum* cv Pentland Ivory-derived *Potato Virus X* resistance locus; ~~resistance to PVS from *S. tuberosum* DW 83-3121,~~ *S. tuberosum* DW 83-3121-derived *Potato Virus S* resistance locus *Ns*; ~~beta-carotene hydroxylase 2 (CHY2) gene,~~ *S. tuberosum* beta-carotene hydroxylase 2 gene *CHY2* (GenBank Acc No HM013963.1); ~~zeaxanthin epoxidase (ZEP) gene,~~ *S. tuberosum* zeaxanthin epoxidase gene *ZEP* (ZEP-1 allele) (GenBank Acc No HM013964.1); *S. tuberosum* zeaxanthin epoxidase gene *ZEP* (ZEP-2 allele) (GenBank Acc No HM013965.1); ~~potato gene for granule-bound starch synthase,~~ *S. tuberosum* granule-bound starch synthase gene *GBSS* (GenBank Acc No X58453.1); ~~gene for beta-fructofuranosidase~~ *S. tuberosum* beta-fructofuranosidase gene *PAIN-1* (GenBank Acc No X70368.1); ~~or potato acid invertase,~~ *S. tuberosum* acid invertase gene (GenBank Acc No AY341425.1); ~~sucrose-phosphate-synthase gene,~~ *S. tuberosum* sucrose-phosphate-synthase gene *sps* (GenBank Acc No EU684230.1); ~~a gene for the putative invertase inhibitor precursor (INH1/INH2),~~ *S. tuberosum* invertase genes *invGE* and *invGF* (GenBank Acc No AJ133765.1); *S. tuberosum* apoplastic invertase gene *InvCD141* (GenBank Acc No HQ110081.1); *S. tuberosum* invertase gene *InvCD111* (GenBank Acc No HQ197977); *S. tuberosum* putative invertase inhibitor precursor gene *INH1* (INH1*A allele) (GenBank Acc No AY864819.1); *S. tuberosum* putative invertase inhibitor precursor gene *INH1* (INH1*B allele)

(GenBank Acc No AY864820.1); *S. tuberosum* invertase inhibitor gene *INH2alpha* (INH2alpha*A1 allele) (GenBank Acc No FJ810205.1); *S. tuberosum* invertase inhibitor gene *INH2alpha* (INH2alpha*A2 allele) (GenBank Acc No FJ810206.1); *S. tuberosum* invertase inhibitor gene *INH2alpha* (INH2alpha*B allele) (GenBank Acc No FJ810207.1); *S. tuberosum* invertase inhibitor gene *INH2alpha* (INH2alpha*C allele) (GenBank Acc No FJ810208.1); S. tuberosum invertase inhibitor gene *INH2alpha* (INH2alpha*D allele) (GenBank Acc No FJ810209.1); *S. tuberosum* putative invertase inhibitor precursor gene *INH2beta* (INH2beta*A1 allele) (GenBank Acc No AY864821.1); ~~gene for tuber polyphenol oxidase PPO,~~ *S. tuberosum* tuber polyphenol oxidase gene *PPO* (POT32 allele) (GenBank Acc No U22921.1); *S. tuberosum* tuber polyphenol oxidase gene *PPO* (POT33 allele) (GenBank Acc No U22922.1); *S. tuberosum* tuber polyphenol oxidase gene *PPO* (POT72 allele) (GenBank Acc No U22923.1); ~~sucrose synthase gene,~~ *S. tuberosum* sucrose synthase gene *StSy2* (GenBank Acc No AY205302.1); ~~chlorogenate inducer gene,~~ *S. tuberosum* chlorogenate inducer gene *Cai1* (GenBank Acc No EU310399.1); ~~gene defined by expressed sequence tags EST724115 and EST717227 for asparagine synthetase,~~ *S. tuberosum* asparagine synthetase expressed sequence tags EST724115 (GenBank Acc No. CK278037.1) and EST717227 (GenBank Acc No. CK271149.1); ~~phosphorylase-L gene,~~ *S. tuberosum* starch phosphorylase gene *L* (GenBank Acc No AF143202.1); ~~gene for R1 protein or glucan water dikinase,~~ *S. tuberosum* glucan water dikinase gene *R1* (GenBank Acc No Y09533.1); ~~all from *S. tuberosum, S. demissum* R2 and R2-like protein genes,~~ *S. demissum* late blight resistance gene *R2* (GenBank Acc No FJ536325.1); *Solanum* sp. AM-3778-16 late blight resistance gene for *R2*-like protein (GenBank Acc No FJ536323.1); ~~*S. hjertingii* R2 late blight resistance protein (Rpi-hjt1.3) gene,~~ *S. hjertingii* R2 late blight resistance gene *Rpi-hjt1.1* (GenBank Acc No. GU563971.1); *S. hjertingii* R2 late blight resistance gene *Rpi-hjt1.2* (GenBank Acc No. GU563972.1; *S. hjertingii* R2 late blight resistance gene *Rpi-hjt1.3* (GenBank Acc No. GU563973.1); ~~ABPT Rpi protein gene,~~ *Solanum* sp. ABPT late blight resistance gene *Rpi* (GenBank Acc No FJ536324.1); ~~*S. venturii* late blight resistance protein genes,~~ *S. venturii* late blight resistance gene *Rpi-vnt1.1* (GenBank Acc No FJ423044.1); *S. venturii* late blight resistance gene *Rpi-vnt1.2* (GenBank Acc No FJ423045.1); *S. venturii* late blight resistance gene *Rpi-vnt1.3* (GenBank Acc No FJ423046.1); ~~*S. bulbocastanum* putative disease resistant protein RGA2 gene,~~ *S. bulbocastanum* putative disease resistant gene *RGA2* (GenBank

Acc No AY336128.1); *S. bulbocastanum* blight resistance protein RPI gene, *S. bulbocastanum* blight resistance gene *RPI* (GenBank Acc No AY426259); *S. bulbocastanum* late blight resistance protein Rpi-blb2 (Rpi-blb2) gene, *S. bulbocastanum* NBS-LRR resistance protein (Rpi-bt1) gene, *S. bulbocastanum* late blight resistance gene *Rpi-bt1* (GenBank Acc No FJ188415.1); *S. tuberosum* nematode resistance protein (Gro1-4) gene, *Solanum spegazzinii* nematode resistance gene *Gro1-4* (GenBank Acc No AY196151.1); *S. tuberosum* disease resistance protein Gpa2 gene, *S. tuberosum* nematode resistance gene *Gpa2* (GenBank Acc No AF195939.1); *S. tuberosum* subsp. *andigena* ADG2/2x(v-2)7a gene controlling resistance to potato Y and A potyviruses, *S. tuberosum* subsp. *andigena* potato Y and A potyvirus resistance gene *ADG2/2x(v-2)7a* (GenBank Acc No. Y15297.1); *S. tuberosum* subsp. *andigena* ry-1 gene for resistance gene-like, exons 1-6, splice variants C38 and C19, *S. tuberosum* subsp. *andigena* potato Y potyvirus resistance gene Y-1 (GenBank Acc No AJ300266.1) and splice variants C38 and C19 thereof; dihydroflavonol 4-reductase gene, *S. tuberosum* dihydroflavonol 4-reductase gene (GenBank Acc No AY289924.1); flavonoid 3',5'-hydroxylase gene and *S. tuberosum* flavonoid 3',5'-hydroxylase (P locus) genes (GenBank Acc Nos. AY675561.1, AY675560.1, AY675558.1 and AY675559.1); gene for the R2R3 MYB transcriptional regulator of anthocyanin 1 production pathway all from *S. tuberosum L.*, *S. tuberosum* anthocyanin 1 gene *an1* (GenBank Acc No AY841129.1); *S. demissum* late blight resistance protein (R1) gene, *S. demissum* late blight resistance gene *R1* (GenBank Acc No AF447489.1); potato late blight resistance protein R3a gene from *S. tuberosum L.*, and *S. demissum* potato late blight resistance gene *R3a* (GenBank Acc No AY849382); *S. bulbocastanum* NBS-LRR resistance protein (Rpi-bt1) gene *S. tuberosum* potato virus X (PVX) resistance gene *rx* (GenBank Acc No AJ011801.1); *S. tuberosum* potato late blight resistance gene *R3b* (GenBank Acc No HQ437670.1); *S. tuberosum* flowering locus gene *T* (FT) (GenBank Acc No GU223211.1); *S. tuberosum* US-W5337.3-derived tuber shape gene locus *Ro*; *S. pinnatisectum* PI 253214-derived *Phytophthora infestans* resistance locus *Rpi1*; *S. berthaultii* PI 473331-derived *Phytophthora infestans* resistance locus *Rpi-ber1*; and *S. microdontum* BGRC 18302(CGN21342)-derived *Phytophthora infestans* resistance locus *Rpi-mcd1*.

2. (Currently amended) A hybrid potato seed resulting from a cross between two essentially homozygous parental potato breeding lines, at least one of which is a plant of a line according to claim 1.

3. (Previously presented) A hybrid potato plant grown from the seed of claim 2.

4. (Currently amended) A tetraploid plant produced by doubling the genome of ~~plants~~ a plant of a line of claim 1.

5. (Currently amended) A method for producing a diploid, fertile, self-compatible and essentially homozygous *Solanum tuberosum* potato line comprising plants having an average tuber yield expressed in grams of fresh weight of at least 200 grams per plant, comprising:

(a) providing a first potato plant, which first potato plant is a plant of a first diploid, self-compatible, fertile and essentially homozygous potato line, wherein said self-compatibility originates from *Solanum chacoense* and is referred to as S-locus inhibitor (*Sli*) gene, as present in a plant of potato lines NCIMB accession number 41663, NCIMB accession number 41664, NCIMB accession number 41665, or NCIMB accession number 41765, representative seeds of said lines having been deposited with the NCIMB, Aberdeen, Scotland under breeders reference AGVD1, AGVD2, AGVD3, and AGVD17 respectively;

(b) providing a second potato plant, wherein said second potato plant is a plant of a diploid or tetraploid *Solanum tuberosum* potato line which may have any level of homozygosity, said line of said second potato plant comprising plants having an average tuber yield expressed in grams of fresh weight of at least 200 grams per plant;

(c) cross pollinating said first and second potato plant to provide seeds and collecting said seeds to thereby provide an offspring generation in the form of a hybrid offspring seed;

(d) growing said hybrid offspring seed into a population of hybrid offspring potato plant and selecting from said population plants producing tubers at a yield expressed in grams of fresh weight of at least 200 grams per plant, and

(e) selfing said hybrid offspring potato plant or backcrossing said hybrid offspring potato plant to a potato plant of said line of said first potato plant, for between 1 to 8 selfings or backcrosses, to thereby provide a diploid, self-compatible and essentially homozygous *Solanum tuberosum* potato breeding line comprising plants having an average tuber yield expressed in grams of fresh weight of at least 200 grams per plant;

(f) said method further comprising the step of introducing into a plant of said diploid, self-compatible and essentially homozygous potato breeding line obtained in step (e) at least one trait or gene selected from the group consisting of ~~resistance to *Phytophthora infestans* from *Solanum bulbocastanum* or *S. demissum*,~~ *S. bulbocastanum* BGRC 8005-derived *Phytophthora infestans* resistance locus *Rpi-blb1*; *S. bulbocastanum* clone PT29/ BAC clone CB3A14-derived *Phytophthora infestans* resistance gene *Rb* (Genbank acc. No. AY303170); *S. bulbocastanum* clone ARD 1197-16-derived *Phytophthora infestans* resistance locus *Rpi-blb2* (Genbank acc. No. DQ122125); *S. bulbocastanum* clone Blb99-256-3-derived *Phytophthora infestans* resistance locus *Rpi-blb3*; *S. bulbocastanum*/*S. tuberosum* somatic hybrid J101-derived *Phytophthora infestans* resistance locus Rpi-bt1; *S. demissum* Black 3053-18-derived *Phytophthora infestans* resistance locus R5; *S. demissum* Black XD2-21-derived *Phytophthora infestans* resistance locus R6; *S. demissum* Black 2182 ef (7)-derived *Phytophthora infestans* resistance locus R7; *S. demissum* Black 2424 a (5)-derived *Phytophthora infestans* resistance locus R8; *S. demissum* Black 2573 (2)-derived *Phytophthora infestans* resistance locus R9; *S. demissum* Black 3681 ad (1)-derived *Phytophthora infestans* resistance locus R10; *S. demissum* Black 5008 ab (6)-derived *Phytophthora infestans* resistance locus R11; ~~resistance to *G. rostochiensis* Ro1 from *S. tuberosum* subsp. *andigena* CPC 1690 or 1673,~~ *S. tuberosum* subsp. *andigena* CPC1673-derived *G. rostochiensis* Ro1 resistance locus H1; ~~resistance to *G. pallida* from *S. tuberosum* subsp. *andigena*,~~ *S. tuberosum* subsp. *andigena* CPC2802-derived *G. pallida* resistance loci *H3* or *GpaIVadg*; ~~resistance to *G. rostochiensis* Ro5-22 and *G. pallida* Pa2-D383 from *S. tuberosum* accession 78-3778,~~ *S. tuberosum* AM78-3778-derived *G. rostochiensis* and *G. pallida* resistance locus *Grp1*; ~~resistance to *G. pallida* Pa2 and Pa3 from *Solanum spegazzinii* and *Solanum tarijense*,~~ *S. spegazzinii* BGRC 8218-15-derived *G. pallida* pathotypes Pa2 and Pa3 resistance locus *Gpa*; *S. tarijense* BGRC 24717-derived *G. pallida* pathotypes Pa2 and Pa3 resistance locus *GpaXI tar 1*; ~~resistance to *G.*~~

~~*pallida* Pa3 from *S. tuberosum* accession 3704-76,~~ *S. tuberosum* 3704-76 (JP)-derived *G. pallida* pathotypes Pa2 and Pa3 resistance loci *Gpa5* and *Gpa6*; ~~resistance to *M. chitwoodi* from *S. bulbocastanum*,~~ *S. bulbocastanum* PI 275187-derived *M. chitwoodi* resistance locus *Rmc1*; ~~resistance to *M. chitwoodi*/*M. fallax* from *S. fendleri*i and *S. hougassii*,~~ *S. fendleri*i BGRC 8083-derived *M. chitwoodi* and *M. fallax* resistance locus *Rmc2*; *S. hougassii* PI 161726-derived *M. chitwoodi* resistance locus *Rmc1(hou)*; ~~resistance to *M. hapla* from *S. chacoense* and *S. tarijense*,~~ *S. chacoense* BGRC 18618-derived *M. hapla* resistance loci *Rmh1* and *Rmh2*; *S. tarijense* BGRC 24717-derived *M. hapla* resistance locus *Rmh3*; ~~resistance to PVX from *S. tuberosum* cv Pentland Ivory,~~ *S. tuberosum* cv Pentland Ivory-derived *Potato Virus X* resistance locus; ~~resistance to PVS from *S. tuberosum* DW 83-3121,~~ *S. tuberosum* DW 83-3121-derived *Potato Virus S* resistance locus *Ns*; ~~beta-carotene hydroxylase 2 (CHY2) gene,~~ *S. tuberosum* beta-carotene hydroxylase 2 gene *CHY2* (GenBank Acc No HM013963.1); ~~zeaxanthin epoxidase (ZEP) gene,~~ *S. tuberosum* zeaxanthin epoxidase gene *ZEP* (ZEP-1 allele) (GenBank Acc No HM013964.1); *S. tuberosum* zeaxanthin epoxidase gene *ZEP* (ZEP-2 allele) (GenBank Acc No HM013965.1); ~~potato gene for granule-bound starch synthase,~~ *S. tuberosum* granule-bound starch synthase gene *GBSS* (GenBank Acc No X58453.1); ~~gene for beta-fructofuranosidase~~ *S. tuberosum* beta-fructofuranosidase gene *PAIN-1* (GenBank Acc No X70368.1); ~~or potato acid invertase,~~ *S. tuberosum* acid invertase gene *Inv* (GenBank Acc No AY341425.1); ~~sucrose-phosphate-synthase gene,~~ *S. tuberosum* sucrose-phosphate-synthase gene *sps* (GenBank Acc No EU684230.1); ~~a gene for the putative invertase inhibitor precursor (INH1/INH2),~~ *S. tuberosum* invertase genes *invGE* and *invGF* (GenBank Acc No AJ133765.1); *S. tuberosum* apoplastic invertase gene *InvCD141* (GenBank Acc No HQ110081.1); *S. tuberosum* invertase gene *InvCD111* (GenBank Acc No HQ197977); *S. tuberosum* putative invertase inhibitor precursor gene *INH1* (INH1*A allele) (GenBank Acc No AY864819.1); *S. tuberosum* putative invertase inhibitor precursor gene *INH1* (INH1*B allele) (GenBank Acc No AY864820.1); *S. tuberosum* invertase inhibitor gene *INH2alpha* (INH2alpha*A1 allele) (GenBank Acc No FJ810205.1); *S. tuberosum* invertase inhibitor gene *INH2alpha* (INH2alpha*A2 allele) (GenBank Acc No FJ810206.1); *S. tuberosum* invertase inhibitor gene *INH2alpha* (INH2alpha*B allele) (GenBank Acc No FJ810207.1); *S. tuberosum* invertase inhibitor gene *INH2alpha* (INH2alpha*C allele) (GenBank Acc No FJ810208.1); *S. tuberosum* invertase inhibitor gene

INH2alpha (INH2alpha*D allele) (GenBank Acc No FJ810209.1); *S. tuberosum* putative invertase inhibitor precursor gene *INH2beta* (INH2beta*A1 allele) (GenBank Acc No AY864821.1); ~~gene for tuber polyphenol oxidase PPO,~~ *S. tuberosum* tuber polyphenol oxidase gene *PPO* (POT32 allele) (GenBank Acc No U22921.1); *S. tuberosum* tuber polyphenol oxidase gene *PPO* (POT33 allele) (GenBank Acc No U22922.1); *S. tuberosum* tuber polyphenol oxidase gene *PPO* (POT72 allele) (GenBank Acc No U22923.1); ~~sucrose synthase gene,~~ *S. tuberosum* sucrose synthase gene *SuSy2* (GenBank Acc No AY205302.1); ~~chlorogenate inducer gene,~~ *S. tuberosum* chlorogenate inducer gene *Cai1* (GenBank Acc No EU310399.1); ~~gene defined by expressed sequence tags EST724115 and EST717227 for asparagine synthetase,~~ *S. tuberosum* asparagine synthetase expressed sequence tags EST724115 (GenBank Acc No. CK278037.1) and EST717227 (GenBank Acc No. CK271149.1); ~~phosphorylase L gene,~~ *S. tuberosum* starch phosphorylase gene *L* (GenBank Acc No AF143202.1); ~~gene for R1 protein or glucan water dikinase,~~ *S. tuberosum* glucan water dikinase gene *R1* (GenBank Acc No Y09533.1); ~~all from *S. tuberosum*, *S. demissum* R2 and R2-like protein genes,~~ *S. demissum* late blight resistance gene *R2* (GenBank Acc No FJ536325.1); *Solanum* sp. AM-3778-16 blight resistance gene for *R2*-like protein (GenBank Acc No FJ536323.1); ~~*S. hjertingii* R2 late blight resistance protein (Rpi-hjt1.3) gene,~~ *S. hjertingii* R2 late blight resistance gene *Rpi-hjt1.1* (GenBank Acc No. GU563971.1); *S. hjertingii* R2 late blight resistance gene *Rpi-hjt1.2* (GenBank Acc No. GU563972.1; *S. hjertingii* R2 late blight resistance gene *Rpi-hjt1.3* (GenBank Acc No. GU563973.1); ~~ABPT Rpi protein gene,~~ *Solanum* sp. ABPT late blight resistance gene *Rpi* (GenBank Acc No FJ536324.1); ~~*S. venturii* late blight resistance protein genes,~~ *S. venturii* late blight resistance gene *Rpi-vnt1.1* (GenBank Acc No FJ423044.1); *S. venturii* late blight resistance gene *Rpi-vnt1.2* (GenBank Acc No FJ423045.1); *S. venturii* late blight resistance gene *Rpi-vnt1.3* (GenBank Acc No FJ423046.1); ~~*S. bulbocastanum* putative disease resistant protein RGA2 gene,~~ *S. bulbocastanum* putative disease resistant gene *RGA2* (GenBank Acc No AY336128.1); ~~*S. bulbocastanum* blight resistance protein RPI gene,~~ *S. bulbocastanum* blight resistance gene *RPI* (GenBank Acc No AY426259); ~~*S. bulbocastanum* late blight resistance protein Rpi-blb2 (Rpi-blb2) gene,~~ *S. bulbocastanum* late blight resistance gene *Rpi-blb2* (GenBank Acc No DQ122125.1); ~~*S. bulbocastanum* NBS-LRR resistance protein (Rpi-bt1) gene,~~ *S. bulbocastanum* late blight resistance gene *Rpi-bt1* (GenBank Acc No FJ188415.1); ~~*S. tuberosum* nematode~~

~~resistance protein (Gro1-4) gene,~~ *Solanum spegazzinii* nematode resistance gene *Gro1-4* (GenBank Acc No AY196151.1); ~~*S. tuberosum* disease resistance protein Gpa2 gene,~~ *S. tuberosum* nematode resistance gene *Gpa2* (GenBank Acc No AF195939.1); ~~*S. tuberosum* subsp. *andigena* ADG2/2x(v-2)7a gene controlling resistance to potato Y and A potyviruses,~~ *S. tuberosum* subsp. *andigena* potato Y and A potyvirus resistance gene *ADG2/2x(v-2)7a* (GenBank Acc No. Y15297.1); ~~*S. tuberosum* subsp. *andigena* ry-1 gene for resistance gene-like, exons 1-6, splice variants C38 and C19,~~ *S. tuberosum* subsp. *andigena* potato Y potyvirus resistance gene Y-1 (GenBank Acc No AJ300266.1) and splice variants C38 and C19 thereof; ~~dihydroflavonol 4-reductase gene,~~ *S. tuberosum* dihydroflavonol 4-reductase gene (GenBank Acc No AY289924.1); ~~flavonoid 3',5'-hydroxylase gene and~~ *S. tuberosum* flavonoid 3',5'-hydroxylase (P locus) genes (GenBank Acc Nos. AY675561.1, AY675560.1, AY675558.1 and AY675559.1); ~~gene for the R2R3 MYB transcriptional regulator of anthocyanin-1-production pathway all from *S. tuberosum L.*,~~ *S. tuberosum* anthocyanin 1 gene *an1* (GenBank Acc No AY841129.1); ~~*S. demissum* late blight resistance protein (R1) gene,~~ *S. demissum* late blight resistance gene *R1* (GenBank Acc No AF447489.1); ~~potato late blight resistance protein R3a gene from *S. tuberosum L.*, and~~ *S. demissum* potato late blight resistance gene *R3a* (GenBank Acc No AY849382); ~~*S. bulbocastanum* NBS-LRR resistance protein (Rpi-blt1) gene~~ *S. tuberosum* potato virus X (PVX) resistance gene *rx* (GenBank Acc No AJ011801.1); *S. demissum* potato late blight resistance gene *R3b* (GenBank Acc No HQ437670.1); *S. tuberosum* flowering locus gene *T* (FT) (GenBank Acc No GU223211.1); *S. tuberosum* US-W5337.3-derived tuber shape gene locus *Ro*; *S. pinnatisectum* PI 253214-derived *Phytophthora infestans* resistance locus *Rpi1*; *S. berthaultii* PI 473331-derived *Phytophthora infestans* resistance locus *Rpi-ber1*; and *S. microdontum* BGRC 18302(CGN21342)-derived *Phytophthora infestans* resistance locus *Rpi-mcd1*.

6. (Previously presented) The method of claim 5, wherein said first potato plant is a plant of potato line NCIMB accession number 41663, NCIMB accession number 41664, NCIMB accession number 41665, or NCIMB accession number 41765, representative seeds of said line having been deposited with the NCIMB, Aberdeen, Scotland under breeders reference AGVD1, AGVD2, AGVD3, and AGVD17 respectively.

7. (Previously presented) A diploid potato breeding line obtained by the method of claim 5.

8. (Currently amended) A method for producing a uniform hybrid potato seed, comprising:

(a) providing a first potato plant of a line according to claim 1,

(b) providing a second potato plant, wherein said second potato plant is a plant of a diploid, fertile, self-compatible and essentially homozygous potato line, and

(c) cross pollinating said first and second potato plant and allowing one of ~~said parent plants~~ either said first or second potato plant to produce berries with seeds and collecting said seeds from said berries to provide an offspring generation in the form of a hybrid offspring seed.

9. (Original) A seed produced by the method of claim 8.

10. (Original) A hybrid potato plant, or part thereof, produced by growing the seed of claim 9.

11. (Original) Pollen or an ovule of the plant of claim 10.

12. (Original) A tuber from the plant of claim 10.

13. (Original) A potato plant having all of the physiological and morphological characteristics of the plant of claim 10.

14. (Previously presented) A tissue culture of the plant of claim 10.

15. (Original) A potato plant regenerated from the tissue culture of claim 14, wherein said regenerated potato plant has all the physiological and morphological characteristics of a potato plant of claim 10.

16. (Original) A method of producing a hybrid potato plant, said method comprising producing a seed according to the method of claim 8 and growing said seed into a potato plant.

17. (Original) A potato plant produced by the method of claim 16, or a tuber thereof.

18. (Currently amended) A set of at least 2 intercrossable diploid, fertile, self-compatible and essentially homozygous potato lines ~~comprising plants~~ according to claim 1.

19. (Previously presented) An elite diploid potato breeding line obtained by the method of claim 5.

REMARKS/ARGUMENTS

Status of Claims

Claims 1-19 were pending in this application. Claims 1, 2, 4, 5, 8 and 18 have been amended.

Basis for this amendment is present throughout the application as filed. For example, support for the amendment to claims 1 and 5 can be found at page 80, lines 2-9 and in Table 10 at pages 83-91.

Claims 2, 4, 8 and 18 have been amended to correct informalities. No new matter is believed to be added herein.

Specification

A. The Examiner has objected to the specification for containing embedded hyperlinks.

The specification has been amended to correct informalities. The specification has been amended to delete the embedded hyperlink and/or other form of browser-executable code. The amendments do not add new matter.

B. The Examiner has further objected to the specification for listing non-patent literature in Table 10 on pages 84-90. Claims 1 and 5 stand rejected under 35 U.S.C. §112 (b) because they lack an art-accepted meaning.

Applicant respectfully disagrees. The potato lines, as claimed in amended claims 1 and 5, comprise at least one agronomically desirable trait or gene. Table 10 provides detailed references to GenBank Accession Numbers for all genes listed. Applicant incorporates all these genes in the said claims. Traits listed in Table 10 are all disease resistance traits, and these are defined as derivable from a specific source. Reference to the non-patent literature in Table 10 is not required to understand the meaning of the claim limitations as amended.

To further support the argument that the terms are not indefinite when explicit reference to the source is made by reference, the following is submitted.

On page 91 of the application as filed, immediately following Table 10, a small table is presented, wherein it is indicated that the traits and genes are known in the art of potato breeding at various levels of detail, allowing the use of any one of four (4) different technical approaches for incorporating the traits or genes in plants of the invention. These technical approaches are indicated as "Class A-Class D".

- When only the biological origin of a trait or gene is known, such trait or gene is identified as "class A". Technical incorporation of the trait or gene may occur by crossing with the source and selection of offspring plants containing the introgression. Such methods are routine when the source is known.

- When both origin and genomic markers are known, such trait or gene is identified as "class B". Technical incorporation of such a trait or gene may occur by crossing and marker-assisted selection. Also, such methods are routine when the source is known.

- When origin, genomic markers and gene sequence are known, such trait or gene is identified as "class C". Technical incorporation of such a trait or gene may occur by crossing and marker-assisted selection or by recombinant or transgenic technology.

- And, finally, when a transgenic plant has already been produced in the art, such a trait or gene is identified as "class D", and technical incorporation of such a trait or gene may occur by crossing and marker-assisted selection or by recombinant or transgenic technology.

Means for producing plants of the invention using any of the genes or traits of class A, B, C or D is expressly detailed in Example 6 "*Introduction of desirable traits*" (pages 70-81 of the description), and in Example 8 (pages 81-93). In fact, in Example 6, the following 4 scenario's are exemplified in detail:

- Incorporation of an agronomically desirable trait from a known source (A);
- Introgression by marker-assisted breeding (B);
- Incorporation of a known gene (C);

- Use of available transgenic plants (D).

For instance, it is described in detail at page 91 of the specification (legend to the table under "A") that, in the instance that only the origin or source (plant accession) of the trait or gene is known, one may (in the instance that the species are non-crossable) "clone the gene and introduce the gene by transformation techniques". Alternatively, (when the species are crossable) one may "make crosses between the donor carrying the trait and the recurrent parent potato plant, select for the desired plant in progenies by (repeated) crossing and selections, self the plant until homozygosity under selection for the desired traits".

Likewise, when selectable markers are known, selection for the desired trait may occur by indirect selection for the marker. The marker-selected plant is ultimately tested for the presence of the desirable trait. (see page 91, legend to the table under "B").

Further, if a gene sequence is known, one may use a similar approach as that provided for A or B, wherein the efficiency of selection is optimized due to the fact that a 100% reliable selectable marker can be generated (see page 91, legend to the table under "C").

Finally, if transgenic plants are already known, the plants of the invention "can be generated by crossing with the transgenic plant carrying the gene for the desired trait. Alternatively, the transgene can be introduced via transformation technologies." (see page 91, legend to the table under "D").

Thus, in order to allow one of skill to produce the plants as claimed, the one of skill must have knowledge of the plant that provides the origin or source of the trait (gene or gene locus). This information is provided in Table 10 and in the references recited therein. It is submitted that the description provides full details on the terms of the claims and explicitly defines the trait in the specification.

Based on the information in Table 10, it is also clear to one of skill in the art when a source plant must be used ("Classes A and B"), or when a gene sequence may be used ("Classes C and D").

With respect to late blight (*Phytophtera infestance*) resistance, it is of note that the nomenclature of late blight genes is as follows: R genes from *S. demissum* are named R1 till R11.

Late blight R genes from other species are abbreviated as Rpi (resistance against *P. infestans*) followed by the abbreviations of the species name (blb from *S. bulbocastanum*, vnt from *Solanum venturii*, etc.). The subsequent number refers to the order of cloning of a particular R gene from that wild species.

Claim 1 has been further amended by specifying that the homozygous potato line is a potato line of the species *Solanum tuberosum* or *Solanum tuberosum* comprising one or more introgression segments of other tuber-bearing *Solanum* species crossable to *Solanum tuberosum*. Basis for this amendment may *inter alia* be found on page 41, lines 8-26, in combination with page 18, lines 1-4 of the application as filed. The material inserted by amendment to claim 1 is the material previously incorporated by reference as indicated in the above table. The amendment does not contain new matter.

Reconsideration and withdrawal of objection are respectfully requested.

Claim Objections

Claims 2 and 4 are objected to because of informalities.

A. Claim 2 is objected to because the subject of claim 1 is a line, not a plant.

The term "of a line" is introduced between "plant" and "according" in claim 2, thereby obviating the objection.

B. Claim 4 is objected to because it recites the plural "plants" rather than reading on a single plant.

Claim 4 is amended to refer to a single "plant".

Reconsideration and withdrawal of objection are respectfully requested.

Rejections under 35 U.S.C. § 112, second paragraph

Claims 1-19 are rejected under 35 U.S.C. § 112 (b), second paragraph, as being indefinite, for failing to particularly point out and distinctly claim the subject matter which Applicant regards as the invention.

A. According to the Examiner, claims 1 and 5 are indefinite in their recitation of numerous terms, for example "*S. demissum* R2 and R2-like protein genes"

Claims 1 and 5 are amended. The trait or gene disclosed in the claims is adequately defined and supported by the specification as filed.

B. According to the Examiner, claims 1 and 5 are indefinite in their recitation of "*S. tuberosum* subsp. andigena ry-1 gene for resistance gene-like, exons 1-6, splice variants C38 and C19".

The phrase "*S. tuberosum* subsp. *andigena* ry-1 gene for resistance gene-like, exons 1-6, splice variants C38 and C19" is replaced with the phrase "*S. tuberosum* subsp. *andigena* potato Y potyvirus resistance gene Y-1 (GenBank Acc No AJ300266.1) and splice variants C38 and C19 thereof". The addition of the specific GenBank accession number eliminates any indefiniteness.

Reconsideration and withdrawal of rejection are respectfully requested.

Rejections under 35 U.S.C. § 112, first paragraph

A. Claims 1-5 and 7-19 are rejected under 35 U.S.C. 112(a) or 35 U.S.C. 112 (pre-AIA), first paragraph, as failing to comply with the written description requirement.

According to the Examiner, the claims are broadly drawn to any diploid, fertile, self-compatible and essentially homozygous potato line. According to the Examiner, the Applicant claims a large genus, whereas the instant application describes only four potato lines to use in the claimed methods that were created from the same line, the IVP07-1001/4 line.

The Examiner further concludes that the Declaration under 37 CFR 1.132 and expert testimonies previously submitted on September 14, 2016 "establish the position that one of skill in the art does not believe Applicant to be in possession of the genus as broadly as claimed."

Applicant respectfully disagrees. In order to expedite prosecution, the claims have been amended to recite a *Solanum tuberosum* potato line.

The Examiner's conclusion regarding Applicant's possession of claimed genus is inter alia based on the Declaration from Dr. Koornneef, which reads as follows:

> "Solynta could successfully obtain essentially homozygous inbred lines by making use of genotypes that are selfcompatible and by choosing the right starting material that apparently is heterozygous for a lower number of deleterious alleles".

According to the Applicant, the position taken by the Examiner appears to be based on a subjective interpretation of the Declaration from Dr. Koornneef. Applicant respectfully points out that this interpretation is incorrect for a number of reasons.

(1) Dr. Koornneef's letter is relied upon to support the non-obviousness of the invention. The letter of Dr. Koornneef is his personal view in response to him becoming aware of the ability of Applicant to produce, for the very first time, a crossbred between two homozygous inbred potatoes of the *S. tuberosum* species. Dr. Koornneef was not asked to give his view on whether he believes the "Applicant was in possession of the genus as broadly as claimed".

(2) The suggestion, coined by the Examiner, that Dr. Koornneef believes the obtainment of the plants as claimed through the use of line IVP07-1001/4 was an isolated and unique event the teaching of which could not be extended to other lines, is not made in the letter of Dr. Koornneef. Rather, Dr. Koornneef indicates that "this provides the basis of generating useful inbred lines that can become parents of F1 hybrids."

(3) The phrasing "making use of genotypes that are self-compatible" clearly indicates that Koornneef did not consider that the teaching would be limited to the use of a particular line as *Sli*-gene donor (e.g. IVP07-1001/4), or a particular type of self-compatibility. The further phrasing "and choosing the right starting material that apparently is heterozygous for a lower number of deleterious alleles" refers to the fact that Applicant has chosen to cross the *Sli*-gene donor lines with diploid *S. tuberosum* potatoes, instead of tetraploid potatoes as was customary in the prior art. It was not until the Applicant successfully produced the homozygous inbred material (instead of ending up with infertile, weak material) that the idea was gaining momentum that the advantageous results obtained by the Applicant was due to the use of diploid starting material and not to a specific tetraploid *S. tuberosum* potato line. The feature that the claimed homozygous plants are diploids is part of the claim, and hence covers the genus as claimed.

In fact, Applicant used multiple *Sli*-gene donor lines (Hosaka) for generating diploid, fertile, self-compatible and essentially homozygous *Solanum* tuberosum potato lines.

The fact that this cannot be repeated for other potato species is not relevant. For example, Dr. Jones indicates that introgression of the *Sli*-gene into *Solanum phureja* did not result in fertile homozygotes that could be useful for hybrid breeding and that could tolerate recurrent selfing:

> "In fact, in the mid 2000s we attempted to produce hybrid potato ourselves. No successes had ever been reported with *S. tuberosum*. In order to work at the diploid level, we back-crossed the *S. chacoense Sli*- gene into *Solanum phureja*. In addition, we selfed the lines that carry the *Sli*- gene. However, we never succeeded in developing any promising material in the form of fertile homozygotes that could be useful for hybrid breeding and that could tolerate recurrent selfing".

The Declaration of Dr. Jones indicates that introgression of the *Sli*-gene into *Solanum phureja* was not successful, but is silent with regard to introgression of the *Sli*-gene into *Solanum tuberosum*, as is presently claimed. As submitted in the Declaration of Lindhout:

> "That attempts to back-cross the *S. chacoense Sli*- gene into *Solanum tuberosum* has not been done as this was considered as useless as the progenies would suffer from the many wild species and deleterious alleles from *S. chacoense*".

Applicant has established that introgression of the *S. chacoense Sli*-gene into *S. tuberosum* is possible, whereby selecting for fertility is a crucial element in an inbreeding program. Inbreeding depression can in fact be overcome at the diploid level of potato. Hence, contrary to all beliefs in the field, diploid, fertile, self-compatible and essentially homozygous *Solanum tuberosum* potato lines can be generated as taught in this application.

It is therefore submitted that the Applicant has sufficiently described a representative number of individual lines in comparison to the claimed *S. tuberosum* plants or lines.

Reconsideration and withdrawal of rejection are respectfully requested.

B. Claims 1-19 are rejected under 35 U.S.C. 112(a) or 35 U.S.C. 112 (pre-AIA), first paragraph, as failing to comply with the written description requirement, as claims 1 and 5 make reference to database accession numbers EST724115 and EST717227.

The reference to expressed sequence tags (EST) accession numbers have been removed from claims 1 and 5, thereby obviating this rejection.

Reconsideration and withdrawal of rejection are respectfully requested.

C. Claims 1-18 are rejected under 35 U.S. C. 112(a) or 35 U.S.C. 112 (pre-AIA), first paragraph, as failing to comply with the enablement requirement. According to the Examiner, the specification does not disclose a repeatable process to obtain the plant and thus it is not apparent if the plant is readily available to the public.

Applicant respectfully bring to the Examiner's attention that a Statement was submitted by the Attorney of record on September 14, 2016 stating that the instant invention will be readily available to the public upon the issuance of a patent and the deposit of the seeds were made under the terms of the Budapest Treaty.

Reconsideration and withdrawal of rejection are respectfully requested.

D. Claims 1-5 and 7-19 are rejected under 35 U.S. C. 112(a) or 35 U.S.C. 112 (pre-AIA), first paragraph, because the specification, while being enabling for the deposited cell lines, does not reasonably provide enablement for forms of self-compatibility as broadly as claimed. According to the Examiner, the specification does not enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the invention commensurate in scope with these claims.

Applicant respectfully disagrees. The amended claims recite a diploid, fertile, self-compatible and essentially homozygous *Solanum tuberosum* potato line. The claimed self-compatibility originates from *S. chacoense* and is referred to as *S-locus* inhibitor (*Sli*) gene. This gene is present in a plant of potato lines under NCIMB accession numbers 41663, 41664, 41665, and 41765.

It is also respectfully pointed out that the phrasing "and choosing the right starting material that apparently is heterozygous for a lower number of deleterious alleles" as in the Declaration of Dr. Koorneef, refers to the fact that Applicant has chosen to cross the *Sli*-gene donor lines with diploid *S. tuberosum* potatoes, instead of tetraploid potatoes as was customary in the art. It was not

until the Applicant had successfully produced the homozygous inbred material (instead of ending up with infertile, weak material) that the idea was gaining momentum that the advantageous results obtained by the Applicant was due to the use of diploid starting material and not to a specific tetraploid *S. tuberosum* potato line. The feature that the homozygous *S. tuberosum* plants comprising the *Sli*-gene are diploids is part of the claim. The disclosed features cover the "genus".

In addition, the Declaration of Dr. Jones indicates that introgression of the *Sli*-gene into *Solanum phureja* did not result in fertile homozygotes that could be useful for hybrid breeding and that could tolerate recurrent selfing.

Both Koorneef and Jones are silent regarding the generation of a diploid, fertile, self-compatible and essentially homozygous *Solanum tuberosum* potato line comprising what is referred to as S-locus inhibitor (*Sli*) gene.

In conclusion, the Applicant has disclosed to the general public the ability to produce lines falling under the scope of the claim and adequately describes working examples of *Solanum tuberosum* plants that are diploid, fertile, self-compatible and essentially homozygous.

Reconsideration and withdrawal of rejection are respectfully requested.

Double Patenting

Claims 1-19 are provisionally rejected on the ground of nonstatutory obviousness-type double patenting as being unpatentable over claims 3 and 9 of copending Application No. 13/504,156.

Applicant respectfully disagrees.

Claims 3 and 9 of the copending Application No. 13/504,156 are drawn to a potato plant and methods of producing a diploid, fertile, self-compatible and essentially homozygous potato line of the species *Solanum tuberosum* or *Solanum tuberosum* comprising introgression segments of other tuber bearing *Solanum* species crossable to *Solanum tuberosum*, said potato line comprising a plant having a tuber yield expressed in grams of fresh weight of at least 200 grams; wherein at least 50% of loci in said plant are homozygous, wherein said self-compatibility originating from *Solanum chacoense* as present in a plant of potato lines NCIMB accession number 41663, NCIMB accession

number 41664, NCIMB accession number 41665, or NCIMB accession number 41765, representative seeds of said lines having been deposited with the NCIMB, Aberdeen, Scotland under breeders reference AGVD1, AGVD2, AGVD3, and AGVD17 respectively, and wherein at least 25% of the plants of said line produce seed-carrying berries.

Amended claims 1 and 5 of the instant application are drawn to a diploid, fertile, self-compatible and essentially homozygous *Solanum tuberosum* potato line comprising an average tuber yield of at least 200 grams per plant, wherein said line comprises at least one or more agronomically desirable traits or genes and wherein said self-compatibility originating from *Solanum chacoense* and is referred to as S-locus inhibitor (*Sli*) gene, as present in a plant of potato lines NCIMB accession number 41663, NCIMB accession number 41664, NCIMB accession number 41665, or NCIMB accession number 41765, representative seeds of said lines having been deposited with the NCIMB, Aberdeen, Scotland under breeders reference AGVD1, AGVD2, AGVD3, and AGVD17 respectively.

An obviousness-type double patenting rejection is appropriate only when the claims of one application are obvious variants of the claims in another application or patent. The pending claims are not obvious variants because there is nothing in claims 1-9 of co-pending Application No. 14/382,952 that fairly suggests the subject matter of amended claim 1.

Reconsideration and withdrawal of the rejection are respectfully requested.

CONCLUSION

Should the Examiner be of the view that an interview would expedite consideration of the application, request is made that the Examiner telephone the applicant's attorney at (617) 535 4453 in order that any outstanding issues be resolved.

Applicant has included the fee for three-month extension of time fee. The Director is hereby authorized to charge any deficiency in the fees filed, asserted to be filed, or which should have been filed herewith (or with any paper hereafter filed in this application by this firm) to our Deposit Account No. 50-1133, under Order No. 091876-0027 from which the undersigned is authorized to draw.

Respectfully submitted,

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McDermott Will & Emery
The McDermott Building
500 North Capitol Street, N.W.
Washington, DC 20001

EXAMINER
BOGGS, RUSSELL T

ART UNIT	PAPER NUMBER
1663	

NOTIFICATION DATE	DELIVERY MODE
12/22/2017	ELECTRONIC

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

mweipdocket@mwe.com
ipdocketboston@mwe.com

Office Action Summary	Application No. 14/112,747	Applicant(s) Lindhout et al.	
	Examiner RUSSELL T BOGGS	Art Unit 1663	AIA Status No

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTHS FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☑ Responsive to communication(s) filed on 15 June 2017 .

☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on _____ .

2a)☐ This action is **FINAL.** 2b) ☑ This action is non-final.

3)☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on _____; the restriction requirement and election have been incorporated into this action.

4)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims*

5) ☑ Claim(s) 1-19 is/are pending in the application.

5a) Of the above claim(s) _____ is/are withdrawn from consideration.

6) ☐ Claim(s) _____ is/are allowed.

7) ☑ Claim(s) 1-19 is/are rejected.

8) ☐ Claim(s) _____ is/are objected to.

9) ☐ Claim(s) _____ are subject to restriction and/or election requirement

* If any claims have been determined allowable, you may be eligible to benefit from the **Patent Prosecution Highway** program at a participating intellectual property office for the corresponding application. For more information, please see http://www.uspto.gov/patents/init_events/pph/index.jsp or send an inquiry to **PPHfeedback@uspto.gov.**

Application Papers

10)☑ The specification is objected to by the Examiner.

11)☐ The drawing(s) filed on _____ is/are: a)☐ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

Priority under 35 U.S.C. § 119

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

Certified copies:

a)☐ All b)☐ Some** c)☐ None of the:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

** See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☐ Notice of References Cited (PTO-892)

2) ☑ Information Disclosure Statement(s) (PTO/SB/08a and/or PTO/SB/08b) Paper No(s)/Mail Date _____.

3) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date _____.

4) ☐ Other: _____.

DETAILED ACTION

Notice of Pre-AIA or AIA Status

The present application is being examined under the pre-AIA first-to-invent provisions.

Status

1. Claims 1-18, filed 18 October 2013, were examined and rejected in the Office action mailed 14 March 2016. Applicant responded on 14 September 2016. Claims 1-19 were examined and rejected on 15 December 2016. Applicant filed an RCE and responded to the final Office action on 15 June 2017.

Claims 1-19 are examined herein.

Withdrawal of Objections and Rejections

2. The objection to the specification the last remaining hyperlink is withdrawn in view of Applicant's amendments.

3. The objections to claims 2 and 4 are withdrawn in view of Applicant's amendments to the claims.

4. The rejection of claims 1 and 5 under 35 U.S.C. 112(b) or 35 U.S.C. 112 (pre-AIA), second paragraph, as being indefinite for recitation of limitations such as "*S. demissum* R2 and R2-like protein genes," in which genes are identified by a variety of terms where the meaning of the terms are indefinite is withdrawn in view of Applicant's amendments to the claims. However the claims remain rejected under 35 USC 112(b).

Examiner's Note

5. 37 CFR 1,52(a)(v) requires that papers submitted to the Office must be "[p]resented in a form having sufficient clarity and contrast between the paper and the writing thereon to permit the direct reproduction of readily legible copies in any number by use of photographic, electrostatic, photo-offset, and microfilming processes and electronic capture by use of digital imaging and optical character recognition." The claim amendments submitted 29 March 2017 do not meet the above requirements. All claim amendments submitted are presented to the Examiner in black and white. The amendments to the claim are made in a font that is represented by gray dots (possibly a colored font?) and cannot be OCRed. Future amendments should conform with the requirements of 37 CFR 1.52.

Citations to the specification herein are abbreviated "Spec."

Claim Interpretation

6. "Essentially homozygous" (e.g. claim 1, line 1) is interpreted in view of the definition in the specification that includes homozygosities as low as 25%. Spec., p. 21.

The limitation "line" is interpreted as being at least two plants that can be selfed. See, e.g., Spec., p. 39, first full para.

Specification

7. The disclosure is objected to because of the following informalities.

The specification lists non-patent literature in Table 10 on pages 84-90. Several of the claim terms in claims 1 and 5 are rejected under 35 USC 112(b) as being indefinite because they lack an art-accepted meaning. Applicant apparently intended to define the claim terms by reference to this table. Thus it is the Office's interpretation that it is Applicant's clear intent to incorporate the essential material cited by reference to the non-patent literature into the claims. The meaning and scope of the claim limitations can only be understood by reference to listed references, however, such an incorporation is improper.

The incorporation of essential material into specification by reference to an unpublished U.S. application, foreign application or patent, to a publication, or to an online database is improper. Applicant is required to amend the specification, if possible, to include the material incorporated by reference, if the material is relied upon to overcome any objection, rejection, or other requirement imposed by the Office. The amendment must be accompanied by a statement executed by the applicant, or a practitioner representing the applicant, stating that the material being inserted is the material previously incorporated by reference and that the amendment contains no new matter. 37 CFR 1.57(f).

Applicant's Arguments

Applicant traverses the rejection in the paper filed 15 June 2017. On page 22 of the response, Applicant argues that Table 10 provides GenBank accession numbers for all genes listed. Response, p. 22.

Applicant further draws attention to a small table on page 91 which instructs the reader how to incorporate the referenced traits and/or genes in plants of the invention.

Id., p. 23. Applicant explains how to use this table to allegedly practice the claimed invention. *Id.*, pp. 23-24.

Applicant mentions the issue regarding R genes from *S. demissum*. *Id.*, pp. 24-25.

Applicant also mentions amendments to claim 1. *Id.*, p. 24.

Response to Applicant's Arguments

Applicant traverses the rejection made on 15 December 2016 in the paper filed 15 June 2017. Applicant's arguments have been fully considered but are not persuasive. Incorporating material by reference to an online database such as GenBank is improper under 37 C.F.R. 1.57(c), as stated above, insofar as the material is essential material.

Referencing class A, B, C and D genes and/or traits merely exacerbates the problem since none appear to be supported by adequate disclosure in the specification or by reference to US Patents or US Patent Publications.

Appropriate correction is requested.

Claim Objections

8. Claims 1, 2, 4, 5, 8 and 15 are objected to because of the following informalities.

Claims 1 and 5 are objected to because, due to the font and/or printer used by Applicant, in numerous places it is not clear whether the amendments use colons and semicolons. For example, in line 12 of claim 1, a colon appears to be used and in line

13 of claim 1, a semicolon appears to be used; however that may not have been Applicant's intent.

Appropriate correction is requested by providing a copy of the claims in accordance with 37 CFR 1,52(a)(v).

Claims 2, 4, 8, and 15 are objected to because they recite the limitation "a line according to claim 1" (claim 1, lines 2-3), "a line of claim 1" (claim 4, line 2), "a line according to claim 1" (claim 8, line 2), and "a potato plant of claim 10" (claim 15, lines 2-3). Since the claims are dependent claims the limitations need to correctly reference their antecedent basis, i.e., be amended to use either "the" or "said" as in "the line according to claim 1."

Improper Markush Group

9. Claims 1-19 are rejected on the judicially-created basis that they contain an improper Markush grouping of alternatives. See *In re Harnisch*, 631 F.2d 716, 721-22 (CCPA 1980) and *Ex parte Hozumi*, 3 USPQ2d 1059, 1060 (Bd. Pat. App. & Int. 1984). The improper Markush groupings in claims 1 and 5 include species of the claimed invention that do not share both a substantial structural feature and a common use that flows from the substantial structural feature. The members of the improper Markush grouping do not share a substantial feature and/or a common use that flows from the substantial structural feature for the following reasons.

Applicant's pending claims read on a large array of individual genes that largely relate to disease resistance. The species encompassed by, e.g. claim 1, constitute an

improper Markush group. The resistance genes, for example, recited in claim 1 represent a diverse group of nucleotide and/or amino acid sequences. There is no evidence on the record that the resistance genes, for example, or their encoded proteins, have any structural similarity. Further, Applicant presents no teachings that such a diverse group of sequences will function equivalently when expressed in a potato plant. Therefore the species of the Markush group lack both a substantial structural feature and a common use that flows from the substantial structural feature

Therefore, one of ordinary skill in the art would not perceive a substantial structural feature and/or a common use that flows from the substantial structural feature in the members of the Markush group.

In response to this rejection, Applicant should amend the claim(s) to recite only a grouping of species that share a substantial structural feature as well as a common use that flows from the substantial structural feature, or present a sufficient showing that the species recited in the alternative of the claims(s) in fact share a substantial structural feature as well as a common use that flows from the substantial structural feature. This is a rejection on the merits and may be appealed to the Board of Patent Appeals and Interferences in accordance with 35 U.S.C. § 134 and 37 CFR 41.31(a)(1).

Claims depending on the claims reciting an improper Markush group are included in this rejection.

Claim Rejections - 35 USC § 112(b)

The following is a quotation of 35 U.S.C. 112(b):

> (B) CONCLUSION.—The specification shall conclude with one or more claims particularly pointing out and distinctly claiming the subject matter which the inventor or a joint inventor regards as the invention.

The following is a quotation of 35 U.S.C. 112 (pre-AIA), second paragraph:

> The specification shall conclude with one or more claims particularly pointing out and distinctly claiming the subject matter which the applicant regards as his invention.

10. Claims 1-19 are rejected under 35 U.S.C. 112(b) or 35 U.S.C. 112 (pre-AIA), second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which the inventor or a joint inventor, or for pre-AIA the applicant regards as the invention.

Claims 1 and 5 are rejected because they recite numerous limitations in which genes, for example, are identified by a variety of terms where the meaning of the terms are indefinite. For example, the first limitation in claim 1, part (f) recites "*S. bulbocastanum* BGRC 8005-derived *Phytophthora infestans* resistance locus Rpi-blb1: *S. bulbocastanum* clone PT29/BAC clone CB3A14-derirved *Phytophthora infestans* resistance gene *Rb* (Genbank acc. No. AY303170)." This term, and the other similar limitations recited in claim 1 and claim 5(f) lack general art-accepted meanings and Applicant does not explicitly define the terms in the specification, for example, by reference to the sequence listing. Applicant provides Table 10 on pages 84 to 90 which cites references from non-patent literature or online databases, however, the incorporation of essential material in the specification by reference to an unpublished U.S. application, foreign application or patent, to a publication, or to an online database is improper. 37 C.F.R. 1.57(c).

Because of the indefiniteness of these terms , the metes and bounds of the claims cannot be determined. Furthermore, the meaning of these terms could arbitrarily change to designate something different during the lifetime of a patent. Thus, one's

ability to determine the metes and bounds of the claim would be impaired. See *In re Hammack*, 427 F .2d 1378, 1382; 166 USPQ 204, 208 (CCPA 1970).

The terms in claims 1 and 5 in question are not listed further due to difficulties in OCRing Applicant's amendments as described *supra*; however recitation of all the specific biological limitations, e.g. the aforementioned "*S. bulbocastanum* BGRC 8005-derived *Phytophthora infestans* resistance locus Rpi-blb1: *S. bulbocastanum* clone PT29/BAC clone CB3A14-derirved *Phytophthora infestans* resistance gene *Rb* (Genbank acc. No. AY303170)" and/or reference to Table 10 in claims 1 and 5(f) are indefinite as claim limitations and render the claims themselves indefinite.

Applicant's Arguments and Response

Applicant traverses the rejection in the paper filed 15 June 2017 on pages 25-26. Applicant argues that, *inter alia*, that the addition of GenBank accession numbers obviates the rejections.

Applicant traverses the rejection made on 15 December 2016 in the paper filed 15 June 2017. Applicant's arguments have been fully considered but are not persuasive. Adding GenBank accession numbers is improper under 37 C.F.R. 1.57(c), as stated above. For example, GenBank accession numbers lead to database entries that can be and frequently are amended.

Furthermore, limitations such as "*S. bulbocastanum* BGRC 8005-derived *Phytophthora infestans* resistance locus Rpi-blb1: *S. bulbocastanum* clone PT29/BAC clone CB3A14-derirved *Phytophthora infestans* resistance gene *Rb* (Genbank acc. No. AY303170)" are indefinite because the relationship of:

(1) "*S. bulbocastanum* BGRC 8005-derived *Phytophthora infestans* resistance locus

Rpi-blb1: *S. bulbocastanum* clone PT29/BAC clone CB3A14-derirved *Phytophthora infestans* resistance gene *Rb*"; and

(2) "Genbank acc. No. AY303170" is indefinite. It is indefinite as to whether the two terms are equivalent or if one is a subset of the other.

Dependent claims are included in this rejection because none provide further limitations obviating this rejection.

Claim Rejections - 35 USC § 112(a), written description

The following is a quotation of 35 U.S.C. 112(a):

> (a) IN GENERAL.—The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same, and shall set forth the best mode contemplated by the inventor or joint inventor of carrying out the invention.

The following is a quotation of 35 U.S.C. 112 (pre-AIA), first paragraph:

> The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same and shall set forth the best mode contemplated by the inventor of carrying out his invention.

11. Claims 1-5 and 7-19 are rejected under 35 U.S.C. 112(a) or 35 U.S.C. 112 (pre-AIA), first paragraph, as failing to comply with the written description requirement. The claim(s) contains subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor or a joint inventor, or for pre-AIA the inventor(s), at the time the application was filed, had possession of the claimed invention.

Applicant traverses the rejection made on 15 December 2016 in the paper filed 15 June 2017. Applicant's arguments have been fully considered but are not persuasive.

Claim 1 is drawn to a diploid, fertile, self-compatible and essentially homozygous *Solanum tuberosum* potato line having an average tuber yield expressed in grams of fresh weight of at least 200 grams per plant; wherein said line additionally comprises 1-6 agronomically desirable traits or genes selected from a Markush group and wherein the self-compatibility originates from *S. chacoense* and is as present in a plant of potato lines NCIMB accession number 41663, NCIMB accession number 41664, NCIMB accession number 41665, or NCIMB accession number 41765, further where representative samples were deposited with the NCIMB under breeders reference AGVD1, AGVD2, AGVD3, and AGVD17.

Claims depending from claim 1 read on a hybrid potato seed produced from a cross between two essentially homozygous parental breeding lines and a potato plant(s) produced therefrom. Claim 8 reads on a method using a plant from claim 1. Claims 9-13 read on plants and plant parts produced by the method of claim 8. Claim 14 reads on a tissue culture produced by the plant of claim 10 and claim 15 reads on a plant produced from the tissue culture of claim 14. Claim 16 reads on a method using a seed produced according to claim 8 to produce a plant; claim 17 reads on a plant or tuber produced according to claim 16. Claim 18 reads on a set of at least 2 intercrossable diploid, fertile, self-compatible and essentially homozygous *S. tuberosum* potato lines according to claim 1.

Claim 5 is a method claim drawn to producing a diploid, fertile, self-compatible and essentially homozygous *Solanum tuberosum* potato line starting with a first potato plant of a first diploid self-compatible, fertile and essentially homozygous potato line wherein the self-compatibility originates from *S. chacoense* and is as present in a plant of potato lines NCIMB accession number 41663, NCIMB accession number 41664, NCIMB accession number 41665, or NCIMB accession number 41765, further where representative samples were deposited with the NCIMB under breeders reference AGVD1, AGVD2, AGVD3, and AGVD17 and crossing it to a second potato plant where the second potato plant has an average tuber yield expressed in grams of fresh weight of at least 200 grams per plant and then selfing or backcrossing to provide a diploid, self-compatible and essentially homozygous *S. tuberosum* potato breeding line comprising plants having an average tuber yield of at least 200 grams per plant, and then introducing into a plant of said line at least one trait or gene selected from a Markush group.

Dependent claim 6 recites specific deposited cell lines and is not rejected under the written description requirement; claim 7 encompasses a diploid potato breeding line obtained by the method of claim 5. Claim 19 reads on an elite diploid potato breeding line obtained by the method of claim 5.

To claim a genus under the written description requirement, Applicant is required to describe a representative number of species to reflect the variation within the genus or structures sufficient to define the genus. The factors to be considered include disclosure of complete or partial structure, physical and/or chemical properties,

functional characteristics, structure/function correlation, methods of making the claimed product, or any combination thereof.

The claimed genera, in particular, include those set forth in the following exemplary claims:

claim 1 – any "diploid, fertile, self-compatible and essentially homozygous *Solanum tuberosum* potato line" wherein the self-compatibility originates from *S. chacoense* and is as present in a plant of potato lines NCIMB accession number 41663, NCIMB accession number 41664, NCIMB accession number 41665, or NCIMB accession number 41765 together with at least one agronomically desirable trait;

claim 5 – any "diploid, fertile, self-compatible and essentially homozygous *Solanum tuberosum* potato line" wherein the self-compatibility originates from *S. chacoense* and is as present in a plant of potato lines NCIMB accession number 41663, NCIMB accession number 41664, NCIMB accession number 41665, or NCIMB accession number 41765 as a starting material in part (a) to produce a "diploid, fertile, self-compatible and essentially homozygous *Solanum tuberosum* potato line" with an added trait or gene.

The above claims, however, are not limited to the self-compatibility genes and/or potato lines described by the deposits. The claims encompass the genus of all self-compatibility that is "as present" in the deposited lines as well as other potato lines. Applicant fails to describe the scope of "as present." For example, the meaning of "as" includes "for instance" and thus the claims also read on other embodiments of self-compatibility. Merriam-Webster, "as", accessed 2 December 2016.

Additionally, claim 13 represents a broad genus. Claim 13 requires all the physiological and morphological characteristics of the plant of claim 10, which is a plant produced by the method of claim 8, which starts with the plant of claim 1. Claim 1 recites only a handful of physiological and morphological characteristics – arguably just the tuber weight, and because claim 8 is a cross with another plant, the physiological and morphological characteristics which will be passed to any given offspring is uncertain and thus the plants produced by the method of claim 8 constitute a large genus.

In the specification, Applicant describes obtaining the IVP07-1001/4 line from publically available germplasm and plants that contain the *Sli* gene and using these plants to breed AGVD1, AGVD2, and AGVD3. Spec., pp. 46-47.

Applicant describes the deposited lines by breeders reference: AGVD1 (NCIMB 41663), AGVD2 (NCIMB 41664), AGVD3 (NCIMB 41665) and AGVD17 (NCIMB 41765). Id., pp. 34-35. Applicant describes a breeding process according to the instant invention starting with AGVD1-3 or AGVD17 on page 39 et seq.

Applicant describes crossing the *Sli* gene donor with various potato clones designated: IVP97-079-9, IVPAA-096-I8 and SH83-92-488 to generate clones D1, D2 and D3. Id., p. 49. Applicant describes creating a line containing the *Sli* gene with enhanced agricultural quality in Example 2, pp. 48-51. Applicant describes creating a molecular marker platform, i.e., SNPs, in Example 3, p. 51 et seq. In Example 4, Applicant describes the development of homozygous diploid potato plants and then F1 hybrid progeny, as well as subsequent generations. Id., p. 60 et seq. Applicant describes the meaning of the claim term "essentially homozygous" on pages 21-22 of

the instant specification and suggests that the term reflects a level of homozygosity that is above 25% or 75%. Id., p. 21-22. Applicant generally describes adding an agronomically desirable trait to a potato genotype on, e.g. page 37.

The genera also include, insofar as they are listed in claims 1 and 5, the genes listed in Table 10. On page 91 of the specification, Applicant indicates that some of the genes listed in Table 10, for example resistance to *M. hapla* from the S. chacoense *RMh1* locus (claim 1, p. 12, l. 12) is only provided by accession, i.e., either the gene must be cloned or the locus crossed in. Such genes are indicated in Table A with an "A." Type "B" entries are described only by reference to genetic markers, but these markers are not described. Type "C" and "D" entries also arguably represent large genera with only a single species arguably described.

Applicant fails to describe a representative number of species in comparison to the size of the genera claimed. In the instant application, Applicant describes only four potato lines to use in the claimed methods or as a starting point to obtain the claimed compositions, and all were created from the same line, the IVP07-1001/4 line. Specification, pp. 46-48. Applicant fails to describe working examples of the broad genus of other potato lines, or embodiments of self-compatibility originating from *S. chacoense*, falling within the scope of the claims. Further, Applicant only describes one parental *S. tuberosum* line and fails to describe examples of claimed potato lines descended from different parental lines.

In the response filed on 14 September 2016, Applicant traverses the rejection under 35 USC 103 and provides background regarding the opinions of artisans in the relevant field. 14 Sept. 2016 Response, pp. 16-22. In support of the argument,

Applicant filed a declaration under 37 CFR 1.132 by Dr. Lindhout with supporting letters. Dr. Lindhout's declaration was formally filed in application serial no. 13/504,156, and the supporting letters were filed in European Application EP2699684 on 04 June 2014 and are copies therefrom. The letters establish the position that one of skill in the art does not believe Applicant to be in possession of the genus as broadly as claimed. For example, on page 2 of his letter, Dr. Koornneef states that

> Ever since Lindhout and his co-workers published their results in Potato Research volume 54 of 2011, showing high yielding hybrids from 87% homozygous F3 inbreds, I have been positively surprised that Solynta has managed to challenge this dogma. Solynta could successfully obtain essentially homozygous inbred lines by making use of genotypes that are selfcompatible and by choosing the right starting material that apparently is heterozygous for a lower number of deleterious alleles. This provides the basis of generating useful inbred lines that can become parents of F1 hybrids.

Letter from Koornneef (2012) p. 2 (underlining added). In another letter supplied in support of Dr. Lindhout's declaration, Dr. Jones states that

> "[I]n the mid 2000s we attempted to produce hybrid potato ourselves. . . . However, we never succeeded in developing any promising material in the form of fertile homozygotes that could be useful for hybrid breeding and that could tolerate recurrent selfing. . . . We considered that this was due to our inability to cross the Sli- self-compatibility locus into good enough or diverse enough diploid *Solanum* germplasm.

Letter from Dr. Jonathan D.G. Jones (2012), p. 1 (underlining added). Both letters applaud Applicant for finding potato lines suitable for the instant invention. Conversely, both letters teach that such lines are exceptional.

Applicant claims a large genus of potato plants and self-compatibility genes. Applicant fails to describe a representative number of species in comparison to the size of the genera claimed. Applicant also fails to describe any structure necessary and/or sufficient for a potato line to be used in the claims, i.e. how to obtain *S. tuberosum* lines

that are "heterozygous for a lower number of deleterious alleles" (Dr. Koornneef) or "good enough or diverse enough diploid *Solanum* germplasm" (Dr. Jones). Applicant also describes only one gene responsible for the self-compatibility or the DNA sequences thereof except by reference to the deposits. Applicant also fails to describe other embodiments of the resistance genes recited in claims 1 and 5 except by improper (*see supra*) reference to the non-patent literature and online databases in Table 10.

The specification therefore fails to provide an adequate written description to support the breadth of the claims. Applicant has not demonstrated possession to one of skill in the art of the instant invention as broadly as claimed at the time of filing, and one of skill in the art would not believe Applicant to be in possession of the genus as broadly as claimed, especially in view of the letters cited above.

Dependent claims are included in this rejection because none provide further limitations obviating this rejection.

Applicant's Arguments

Applicant argues against the rejection in the response filed on 15 June 2017. Response, pp. 26-28. Applicant attempts to argue that Dr. Koornneef's letter is not relevant to the current rejection. *Id.*, pp. 26-27. Applicant then addresses Dr. Jones letter. *Id.*, p. 28.

Response to Applicant's Arguments

At issue is whether a representative number of *S. tuberosum* potato lines have been described relative to the size of the genus claimed. It is customary in written description rejections to discuss species / genus relationships, however the word

"species" is ambiguous in the instant rejection. At issue is whether a representative number of 'species' of *S. tuberosum* lines, i.e. where *S. tuberosum* is arguably a single species in the biological sense, have been described. Applicant also fails to describe a representative number species (patent sense) in comparison to the size of the genus claimed for a number of other genera.

Turning to Applicant's arguments, the reason behind Dr. Koornneef's letter is irrelevant. Dr. Koornneef is presented by Applicant as an expert in the field and Dr. Koornneef expresses surprise at Applicant's success. This is relevant to the instant rejection. Applicant's attorney's argument attempting to distinguish the teachings in Dr. Koornneef's letter is not persuasive. Finally, regarding point (3) in Applicant's arguments regarding Dr. Koornneef's letter, the primary issue above is "choosing the right starting material that apparently is heterozygous for a lower number of deleterious alleles" in combination with the self-compatibility used by Applicant. Response, p. 26 (quoting Dr. Koornneef). Applicant only describes a single embodiment of a *S. tuberosum* line, and the deposits, when compared to the size of the genus claimed.

Dr. Jones also teaches that it is difficult to obtain "fertile homozygotes that could be useful hybrid breeding and the could tolerate recurrent selfing." Response, p. 26 (quoting Dr. Jones).

Thus Applicant's argument fails to persuade.

Claim Rejections - 35 USC § 112(a); improper incorporation by reference

12. Claims 1-19 are rejected under the written description requirement because claims 1 and 5 make reference to database accession numbers numerous places in the claims, for example, Genbank acc. No. AY303170 in line 13 of claim 1. There are approximately 55 references to database accession numbers in claim 1. It is the Office's interpretation that it is Applicant's clear intent to incorporate the essential material cited by reference to the accession numbers into the claims. The meaning and scope of the claim limitations can only be understood by reference to the accession numbers, however, such an incorporation is improper.

The incorporation of essential material in the claims by reference to an unpublished U.S. application, foreign application or patent, to a publication, or to an online database is improper. Applicant is required to amend the claims to include the material incorporated by reference, if the material is relied upon to overcome any objection, rejection, or other requirement imposed by the Office. The amendment must be accompanied by a statement executed by the applicant, or a practitioner representing the applicant, stating that the material being inserted is the material previously incorporated by reference and that the amendment contains no new matter. 37 CFR 1.57(f).

Dependent claims are included in this rejection because none provide further limitations obviating this rejection.

Included in the list of accession numbers is EST717227 which Applicant asserted was removed from the claim on page 29 of the response filed on 15 June 2017.

Claim Rejections - 35 USC § 112(a); new matter

13. Claims 1-19 are rejected under the written description requirement because the amendments to claims 1 and 5 contain new matter. For example, there is no evidence that any of the following terms appear in the specification, abstract or claims as filed:

BGRC 8005

PT29

Genbank acc. No. AY303170

1197

INH1*A or INHI*B

etc.

Therefore the appearance of these terms and others in the claims comprise NEW MATTER. In response to this rejection, Applicant is required to point to support for the claims or to cancel the new matter.

Dependent claims are included in this rejection because none supply limitations obviating this rejection.

Claim Rejections - 35 USC § 112(a); enablement re: deposits

14. Claims 1-19 are rejected under 35 U.S.C. 112(a) or 35 U.S.C. 112 (pre-AIA), first paragraph, as failing to comply with the enablement requirement. The claim(s) contains subject matter which was not described in the specification in such a way as to enable one skilled in the art to which it pertains, or with which it is most nearly connected, to make and/or use the invention.

Applicant traverses the rejection made on 15 December 2016 in the paper filed 15 June 2017. Applicant's arguments have been fully considered but are not persuasive.

The invention appears to employ novel plants. Since the plant is essential to the claimed invention it must be obtainable by a repeatable method set forth in the specification or otherwise be readily available to the public. If the plant is not so obtainable or available, the requirements of 35 USC § 112 may be satisfied by a deposit of the seeds. A deposit of 2500 seeds of each of the claimed embodiments is considered sufficient to ensure public availability.

The specification does not disclose a repeatable process to obtain the plant and thus it is not apparent if the plant is readily available to the public.

(a) If a deposit is made under the terms of the Budapest Treaty, then a statement, affidavit or declaration by Applicant, or a statement by an attorney of record over his or her signature and registration number, or someone empowered to make such a statement, stating that the instant invention will be irrevocably and without restriction released to the public upon the issuance of a patent, would satisfy the deposit requirement made herein.

(b) If a deposit has not been made under the Budapest Treaty, then in order to certify that the deposit meets the criteria set forth in 37 CFR 1.801-1.809 and MPEP 2402-2411.05, Applicant may provide assurance of compliance by statement, affidavit or declaration, or by someone empowered to make the same, or by a statement by an attorney of record over his or her signature and registration number showing that:

(i) during the pendency of this application, access to the invention will be afforded to the Commissioner upon request;

(ii) all restrictions upon availability to the public will be irrevocably removed upon granting of the patent in accordance with 37 CFR § 1.808(a)(2);

(iii) the deposit will be maintained in a public depository for a period of 30 years or 5 years after the last request or for the effective life of the patent, whichever is longer;

(iv) a test of the viability of the biological material at the time of deposit (see 37 CFR § 1.807); and,

(v) the deposit will be replaced if it should ever become inviable.

In addition, the identifying information set forth in 37 CPR 1.809(d) should be added to the specification, if not already present. See 37 CPR 1.801 - 1.809 (MPEP 2401-2411.05) for additional explanation of these requirements.

Applicant has deposited seeds (see e.g. pages 34-35 of the instant specification). The deposit was made under the terms of the Budapest treaty. Response, p. 15. Examiner also notes that, *inter alia*, a statement, affidavit or declaration by Applicant, or a statement by an attorney of record over his or her signature and registration number, or someone empowered to make such a statement, stating that the instant invention will be irrevocably and without restriction released to the public upon the issuance of a patent is required.

Applicant's Arguments and Response

On page 29 of the response filed on 15 June 2017, Applicant appears to refer to the statement on page 15 of 14 September 2016 although Applicant fails to provide a precise citation.

As stated in the Office action on 15 December 2016, Applicant, however, has not affirmed that the deposits, e.g. recited in claim 1, will be irrevocably and without restriction released to the public upon the issuance of a patent. Thus Applicant has failed to perfect the deposit. Therefore the rejection is maintained.

Claim Rejections - 35 USC § 112(a); enablement re: "A, B, C, and D" listings

15. Claims 1-19 are rejected under 35 U.S.C. 112(a) or 35 U.S.C. 112 (pre-AIA), first paragraph, as failing to comply with the enablement requirement. The claim(s) contains subject matter which was not described in the specification in such a way as to enable one skilled in the art to which it pertains, or with which it is most nearly connected, to make and/or use the invention.

This is a new rejection in addition to the rejection of record involving the deposits.

The invention appears to employ novel plants as a source for the genes required in claims 1 and 5. *See* e.g., Spec., p. 91. Since the plant/plants is/are essential to the claimed invention it must be obtainable by a repeatable method set forth in the specification or otherwise be readily available to the public. If the plant is not so obtainable or available, the requirements of 35 USC § 112 may be satisfied by a deposit

of the seeds. A deposit of 2,500 seeds of each of the claimed embodiments is considered sufficient to ensure public availability.

The specification does not disclose a repeatable process to obtain the plant and thus it is not apparent if the plant is readily available to the public.

(a) If a deposit is made under the terms of the Budapest Treaty, then a statement, affidavit or declaration by Applicant, or a statement by an attorney of record over his or her signature and registration number, or someone empowered to make such a statement, stating that the instant invention will be irrevocably and without restriction released to the public upon the issuance of a patent, would satisfy the deposit requirement made herein.

(b) If a deposit has not been made under the Budapest Treaty, then in order to certify that the deposit meets the criteria set forth in 37 CFR 1.801-1.809 and MPEP 2402-2411.05, Applicant may provide assurance of compliance by statement, affidavit or declaration, or by someone empowered to make the same, or by a statement by an attorney of record over his or her signature and registration number showing that:

(i) during the pendency of this application, access to the invention will be afforded to the Commissioner upon request;

(ii) all restrictions upon availability to the public will be irrevocably removed upon granting of the patent in accordance with 37 CFR § 1.808(a)(2);

(iii) the deposit will be maintained in a public depository for a period of 30 years or 5 years after the last request or for the effective life of the patent, whichever is longer;

(iv) a test of the viability of the biological material at the time of deposit (see 37 CFR § 1.807); and,

(v) the deposit will be replaced if it should ever become inviable.

In addition, the identifying information set forth in 37 CPR 1.809(d) should be added to the specification, if not already present. See 37 CPR 1.801 - 1.809 (MPEP 2401-2411.05) for additional explanation of these requirements.

On page 91 of the specification, Applicant indicates that some of the genes listed in Table 10, for example resistance to *M. hapla* from the S. chacoense *RMh1* locus (claim 1, p. 12, l. 12) is only provided by accession, i.e., either the gene must be cloned or the locus crossed in. Such genes are indicated in Table A with an "A." Therefore to practice these "A" embodiments of the invention, the plant must be made available by deposit or, as set forth above, "must be obtainable by a repeatable method set forth in the specification or otherwise be readily available to the public." There is no evidence on the record that a deposit of the lines required by "A" entries were made, that they are obtainable by a repeatable method set forth in the specification, or are otherwise readily available to the public. Therefore those at least those citations in Table 10 that are referenced with an "A" and are placed as limitations in claims 1 and/or 5 are not enabled.

Type "B" entries are also included in this rejection because the gene required for resistance is only characterized by diagnostic markers and further, those diagnostic markers are neither described nor taught.

Claim Rejections - 35 USC § 112(a); re: Ex parte Jackson

16. Claims 1-5 and 7-19 are rejected under 35 U.S.C. 112(a) or 35 U.S.C. 112 (pre-AIA), first paragraph, because the specification, while being enabling for the deposited cell lines, does not reasonably provide enablement for forms of self-compatibility as broadly as claimed. The specification does not enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the invention commensurate in scope with these claims.

Applicant traverses the rejection made on 15 December 2016 in the paper filed 15 June 2017. Applicant's arguments have been fully considered but are not persuasive.

The invention appears to employ plants with novel characteristics. Since these plant characteristics are essential to the claimed invention it must be obtainable by a repeatable method set forth in the specification or otherwise be readily available to the public. If the plant is not so obtainable or available, the requirements of 35 USC § 112 may be satisfied by a deposit of the seeds. Deposits under NCIMB accession numbers 41663, 41664, 41665 and 41765 have been made. However the claims are not drawn solely to the deposits.

Claim 1 is drawn to a diploid, fertile, self-compatible and essentially homozygous *S. tuberosum* potato line having an average tuber yield expressed in grams of fresh weight of at least 200 grams per plant; wherein said line additionally comprises 1-6 agronomically desirable traits or genes selected from a Markush group and wherein the self-compatibility originates from *S. chacoense* and is as present in a plant of potato

lines NCIMB accession number 41663, NCIMB accession number 41664, NCIMB accession number 41665, or NCIMB accession number 41765, further where representative samples were deposited with the NCIMB under breeders reference AGVD1, AGVD2, AGVD3, and AGVD17.

Claims depending from claim 1 read on a hybrid potato seed produced from a cross between two essentially homozygous parental breeding lines and a potato plant(s) produced therefrom. Claim 8 reads on a method using a plant from claim 1. Claims 9-13 read on plants and plant parts produced by the method of claim 8. Claim 14 reads on a tissue culture produced by the plant of claim 10 and claim 15 reads on a plant produced from the tissue culture of claim 14. Claim 16 reads on a method using a seed produced according to claim 8 to produce a plant; claim 17 reads on a plant or tuber produced according to claim 16. Claim 18 reads on a set of at least 2 intercrossable diploid, fertile, self-compatible and essentially homozygous *S. tuberosum* lines according to claim 1.

Claim 5 is a method claim drawn to producing a diploid, fertile, self-compatible and essentially homozygous *S. tuberosum* line starting with a first potato plant of a first diploid self-compatible, fertile and essentially homozygous potato line wherein the self-compatibility originates from *S. chacoense* and is as present in a plant of potato lines NCIMB accession number 41663, NCIMB accession number 41664, NCIMB accession number 41665, or NCIMB accession number 41765, further where representative samples were deposited with the NCIMB under breeders reference AGVD1, AGVD2, AGVD3, and AGVD17 and crossing it to a second potato plant where the second potato plant has an average tuber yield expressed in grams of fresh weight of at least 200

grams per plant and then selfing or backcrossing to provide a diploid, self-compatible and essentially homozygous *S. tuberosum* breeding line comprising plants having an average tuber yield of at least 200 grams per plant, and then introducing into a plant of said line at least one trait or gene selected from a Markush group.

Dependent claim 6 recites specific deposited cell lines and is not rejected under the written description requirement; claim 7 encompasses a diploid potato breeding line obtained by the method of claim 5. Claim 19 reads on an elite diploid potato breeding line obtained by the method of claim 5.

The claimed genera, in particular, include those set forth in the following exemplary claims:

claim 1 – any "diploid, fertile, self-compatible and essentially homozygous *Solanum tuberosum* potato line" wherein the self-compatibility originates from *S. chacoense* and is as present in a plant of potato lines NCIMB accession number 41663, NCIMB accession number 41664, NCIMB accession number 41665, or NCIMB accession number 41765 together with at least one agronomically desirable trait;

claim 5 – any "diploid, fertile, self-compatible and essentially homozygous *Solanum tuberosum* potato line" wherein the self-compatibility originates from *S. chacoense* and is as present in a plant of potato lines NCIMB accession number 41663, NCIMB accession number 41664, NCIMB accession number 41665, or NCIMB accession number 41765 as a starting material in part (a) to produce a diploid, fertile, self-compatible and essentially homozygous *S. tuberosum* line with an added trait or gene.

The above claims, however, are not limited to the self-compatibility gene and potato lines described by the deposited accessions. The claims encompass the genus

of all self-compatibility genes that are "as present" in the deposited lines. Applicant fails to describe the scope of "as present." For example, the meaning of "as" includes "for instance" and thus the claims also read on other embodiments of self-compatibility genes and potato lines. Merriam-Webster, "as", accessed 2 December 2016.

The claims also read on all "diploid, fertile, self-compatible and essentially homozygous *Solanum tuberosum* potato" lines but only one is taught.

The Federal Circuit in *In re Wands* lists eight considerations for determining whether or not undue experimentation would be necessary to practice an invention. *In re Wands*, 858 F2d 731, 8 USPQ2d 1400, 1406 (Fed. Cir. 1988). These factors are: the quantity of experimentation necessary, the amount of direction or guidance presented, the presence or absence of working examples of the invention, the nature of the invention, the state of the prior art, the relative skill of those in the art, the predictability or unpredictability of the art, and the breadth of the claims. *Id*.

In the specification, Applicant teaches obtaining the IVP07-1001/4 line from publically available germplasm and plants that contain the *Sli* gene and using these plants to breed AGVD1, AGVD2, and AGVD3. Specification ("Spec."), pp. 46-47. Applicant teaches that lines were deposited and that the deposited lines are known by their breeders reference: AGVD1 (NCIMB 41663), AGVD2 (NCIMB 41664), AGVD3 (NCIMB 41665) and AGVD17 (NCIMB 41765). Id., pp. 34-35. Applicant teaches a breeding process according to the instant invention starting with AGVD1-3 or AGVD17 on page 39 et seq.

Applicant teaches crossing the *Sli* gene donor with various potato clones designated IVP97-079-9, IVPAA-096-I8 and SH83-92-488 to generate clones D1, D2

and D3. Id., p. 49. Applicant teaches creating a line containing the *Sli* gene with enhanced agricultural quality in Example 2, pp. 48-51. Applicant teaches creating a molecular marker platform, i.e., SNPs, in Example 3, p. 51 et seq. In Example 4, Applicant teaches the development of homozygous diploid potato plants and then F1 hybrid progeny, as well as subsequent generations. Id., p. 60 et seq. Applicant teaches the meaning of the claim term "essentially homozygous" on pages 21-22 of the instant specification and suggests that the term reflects a level of homozygosity that is above 25% or 75%. Id., p. 21-22. Applicant generally teaches adding an agronomically desirable trait to a potato genotype on, e.g. page 37.

The art generally teaches this breeding strategy, or similar strategies. For example, Jansky teaches first obtaining homozygous diploids using a self-incompatibility inhibitor. J Jansky, "Breeding, Genetics, and Cultivar Development," Advances in Potato Chemistry and Technology, ed. J. Singh & L. Kaur, pp. 27-62 (June, 2009), p. 46. Jansky teaches that "[h]ighly homozygous inbreds could be also be used for heterosis breeding. Jansky, p. 46. Jansky teaches that such an approach would be difficult. *Id*.

Phumichai et al. confirms the approach of Jansky, and documents the use of the *Sli* gene in the approach. Phumichai et al., Genome 48:977-84 (2005), abstract.

> Potato (Solanum tuberosum) is an auto- [sic] or segmental allo-tetraploid showing tetrasomic inheritance, which results in complicated genetic segregation. Since potatoes are propagated vegetatively, highly heterotic genotypes can be maintained, which facilitates the use of heterosis, an essential factor in potato breeding, particularly for tuber yield. Alternatively, open-pollinated true potato seed (TPS) can be used as a low-cost planting material for potato production.

> Highly homozygous inbreds, once amply produced, would be highly useful for the development of TPS varieties with genetic uniformity, as well as for the investigation of heterosis."
>
> "A scheme for hybrid breeding using homozygous diploids and subsequent somatic hybridization has been proposed, for which the availability of highly homozygous diploid (or pure) lines is a key for success. In considering the use of pure lines for the development of genetically uniform [true potato seed] varieties, self-fertile, seed-propagated lines are highly desirable, and simple techniques to develop such lines are necessary." .

Id., pp. 977-78 (citations omitted).

In the response filed on 14 September 2016, Applicant traverses the rejection of record at that time under 35 USC 103 and argues that one of skill in the art would not have a reasonable expectation of success. Response, pp. 16-22. As part of the argument, Applicant reviews the scope and content of the prior art, including the references cited above. For example, Applicant cites to a publication from Watanabe et al. (Breed Sci 46:329-36 (1996)) as standing for "homozygous inbred lines have not been obtained at the diploid level due to both inbreeding depression and self-incompatibility." Response, p. 17. Applicant also cites to Jansky as downplaying the possibility of hybrid breeding as 'commercially unfeasible due to "dramatic reductions in fertility and vigor.'" Response, p. 17 (quoting Jansky, p. 46). Applicant also argues that Uijtewaal et al. (Euphytica, 36: 745-753, 1987) confirms this teaching. Response, p. 17 & p. 19. Applicant concludes the argument with citation of additional material supporting their position that one of skill in the art would not have a reasonable expectation of success in combining the art cited in the rejection under 35 USC 103.

In support of the argument, Applicant also filed a declaration under 37 CFR 1.132 by Dr. Lindhout with supporting letters. Dr. Lindhout's declaration was formally filed in

application serial no. 13/504,156, and the supporting letters were initially filed in European Application EP2699684 (04 June 2014) and are copies therefrom. The letters teach that the success of Applicant's invention, as broadly as claimed, was unpredictable. For example, on page 2 of his letter, Dr. Koornneef states that

> Ever since Lindhout and his co-workers published their results in Potato Research volume 54 of 2011, showing high yielding hybrids from 87% homozygous F3 inbreds, I have been positively surprised that Solynta has managed to challenge this dogma. Solynta could successfully obtain essentially homozygous inbred lines by making use of genotypes that are selfcompatible and by choosing the right starting material that apparently is heterozygous for a lower number of deleterious alleles. This provides the basis of generating useful inbred lines that can become parents of F1 hybrids.

Letter from Koornneef (2012) p. 2 (underlining added). Earlier, Dr. Koornneef also states that "to start a project aiming at developing a good performing homozygous potato plant for the purpose of hybrid production is a daring undertaking with very little chance of success. *Id.*

In another letter supplied in support of Dr. Lindhout's declaration, Dr. Jones states that

> "[I]n the mid 2000s we attempted to produce hybrid potato ourselves. . . . However, we never succeeded in developing any promising material in the form of fertile homozygotes that could be useful for hybrid breeding and that could tolerate recurrent selfing. . . . We considered that this was due to our inability to cross the Sli- self-compatibility locus into good enough or diverse enough diploid *Solanum* germplasm.

Letter from Dr. Jonathan D.G. Jones (2012), p. 1 (underlining added).

Applicant claims a large genus of potato plants. Jansky and Spooner et al. describes the breadth of the of the claimed genus as extremely large. See, e.g., Jansky, "Breeding, Genetics, and Cultivar Development," Advances in Potato Chemistry and Technology, ed. J. Singh & L. Kaur, pp. 27-62 (June, 2009), pp. 27-29; Spooner et

al., Bot Rev 80:283-383 (2014), Table 1, pp. 286-94. Applicant's claimed invention reads on any potential potato line that is a "diploid, fertile, self-compatible and essentially homozygous *Solanum tuberosum* potato line" where the self-compatibility is "as present" in the deposited samples.

In view of Applicant's arguments in response to the rejection under 35 USC 103 and Dr. Lindhout's supporting letters, success in practicing Applicant's claimed invention would be unpredictable and would depend on the genotype of the potato lines. Therefore, extensive guidance would be required to enable the invention as broadly as claimed. Such guidance is not provided.

The specification therefore does not enable one skilled in the art to make and use members of the species of the claimed genus other than the deposited lines. See, for example, MPEP 2164.06(a)(II), citing to *Ex parte Jackson*, 217 USPQ 804, 806 (Bd. App. 1982). The issue before the Board in *Ex parte Jackson* was:

> The issue squarely raised by this rejection is whether or not a description of several newly discovered strains of bacteria having one particularly desirable metabolic property in terms of the conventionally measured culture characteristics and a number of metabolic and physiological properties would enable one of ordinary skill in the relevant art to independently discover additional strains having the same specific, desirable metabolic property, i.e., the production of a particular antibiotic.
>
> To state the issue somewhat differently, it is whether a verbal description of a new species would enable one of ordinary skill in the relevant art to obtain strains of that species over and above the specific strains made available through deposit in one of the recognized culture depositories.

Ex parte Jackson, 217 USPQ 804, 806 (Bd. App. 1982). In the instant application, the issue is similar to the one before the Board in that Applicant made deposits with unique characteristics but not taught how to identify other lines. In *Ex parte Jackson*, the Board held that the deposit, without a detailed explanation of how to obtain more species from

nature, did not enable the generic claims. *Id.*, p. 808. The board found that "the degree of experimentation involved in locating new [organisms] apart from deposited cultures is undue in light of the enablement requirement of 35 U.S.C. 112." *Id.* The *Jackson* decision was considered and distinguished by the Federal Circuit in the *Wands* decision in view of the routine experimentation required to obtain hybridomas. *In re Wands*, pp. 1403-04.

The claimed but not enabled genera also include, insofar as they are listed in claims 1 and 5, the genes listed in Table 10. On page 91 of the specification, Applicant indicates that some of the genes listed in Table 10, for example resistance to *M. hapla* from the S. chacoense *RMh1* locus (claim 1, p. 12, l. 12) is only provided by accession, i.e., either the gene must be cloned or the locus crossed in. Such genes are indicated in Table A with an "A." Type "B" entries are described only by reference to genetic markers, but these markers are not taught. Type "C" and "D" entries also arguably represent large genera with only a single species taught.

Therefore, in view of the *Ex parte Jackson* decision, the generic scope of claims 1 and 5, reading on the genus of as set forth above is not enabled. Obtaining further species falling within the scope of the generic claim would require undue experimentation.

Dependent claims are included in this rejection because none provide further limitations obviating this rejection.

Applicant's Arguments

Applicant traverses the rejection on pages 29-30 of the response. Applicant reviews the teachings of the specification. Response, pp. 29-30. Applicant mentions

the letters of Dr. Koornneef and Dr. Jones. *Id.*, p. 30. Applicant argues that since one embodiment is taught the invention is fully enabled – "Applicant has disclosed to the general public the ability to produce lines falling under the scope of the claim". *Id.*

Response to Applicant's Arguments

The rejection stands as set forth above.

Applicant fails to enable the invention as broadly claimed. For example, the Supreme Court stated that "[h]he basic quid pro quo contemplated by the Constitution and the Congress for granting a patent monopoly is the benefit derived by the public from an invention with substantial utility. Unless and until a process is refined and developed to this point--where specific benefit exists in currently available form--there is insufficient justification for permitting an applicant to engross what may prove to be a broad field." *Brenner v. Manson*, 383 U.S. 519, 148 USPQ 689, 695 (1966). Applicant teaches a single embodiment but only the single embodiment is taught. In view of, for example, the surprise of Dr. Koornneef and Dr. Jones, Applicant fails to provide the public with the knowledge of how to practice Applicant's invention with the exception of the embodiments of the deposited lines.

Double Patenting

The nonstatutory double patenting rejection is based on a judicially created doctrine grounded in public policy (a policy reflected in the statute) so as to prevent the unjustified or improper timewise extension of the "right to exclude" granted by a patent and to prevent possible harassment by multiple assignees. A nonstatutory obviousness-type double patenting rejection is appropriate where the conflicting claims are not identical, but at least one examined application claim is not patentably distinct from the reference claim(s) because the examined application claim is either anticipated by, or would have been obvious over, the reference claim(s). See, e.g., *In re Berg*, 140

F.3d 1428, 46 USPQ2d 1226 (Fed. Cir. 1998); *In re Goodman*, 11 F.3d 1046, 29 USPQ2d 2010 (Fed. Cir. 1993); *In re Longi*, 759 F.2d 887, 225 USPQ 645 (Fed. Cir. 1985); *In re Van Ornum*, 686 F.2d 937, 214 USPQ 761 (CCPA 1982); *In re Vogel*, 422 F.2d 438, 164 USPQ 619 (CCPA 1970); and *In re Thorington*, 418 F.2d 528, 163 USPQ 644 (CCPA 1969).

A timely filed terminal disclaimer in compliance with 37 CFR 1.321(c) or 1.321(d) may be used to overcome an actual or provisional rejection based on a nonstatutory double patenting ground provided the conflicting application or patent either is shown to be commonly owned with this application, or claims an invention made as a result of activities undertaken within the scope of a joint research agreement.

Effective January 1, 1994, a registered attorney or agent of record may sign a terminal disclaimer. A terminal disclaimer signed by the assignee must fully comply with 37 CFR 3.73(b).

The USPTO internet Web site contains terminal disclaimer forms which may be used. Please visit http://www.uspto.gov/forms/. The filing date of the application will determine what form should be used. A web-based eTerminal Disclaimer may be filled out completely online using web-screens. An eTerminal Disclaimer that meets all requirements is auto-processed and approved immediately upon submission. For more information about eTerminal Disclaimers, refer to http://www.uspto.gov/patents/process/file/efs/guidance/eTD-info-I.jsp.

17. Claims 1-19 are provisionally rejected on the ground of nonstatutory obviousness-type double patenting as being unpatentable over claims 3 and 9 of copending Application No. 13/504,156. Although the conflicting claims are not identical, they are not patentably distinct from each other. This is a provisional double patenting rejection since the conflicting claims have not yet been patented.

Applicant traverses the rejection made on 15 December 2016 in the paper filed 15 June 2017. Applicant's arguments have been fully considered but are not persuasive.

Claims 3 and 9 of the 13/504,156 application relate to potato lines/plants of a "diploid, fertile, self-compatible and essentially homozygous *Solanum tuberosum* potato

line" comprising agronomically desirable traits and methods using such lines to create plants comprising agronomically desirable traits. The specifications of the two applications teach the same deposited potato lines. Claims 3 and 9 of the 13/504,156 application claim a broad genus, including, e.g., pest/disease resistance, while the instant claim set encompass a sub-genus of the claimed genus listing numerous specific examples of genes associated with, e.g., pest/disease resistance. Instant claims 1 and 5 generally recite various embodiments of disease resistance, e.g. claim 1, lines 11 et seq. Therefore the instant application reads on a sub-genus of the genus claimed in the 13/504,156 application.

Applicant's Arguments and Response

Applicant traverses the rejection on pages 30-31 of the response. Applicant effectively only presents the scope of the claims in Applicant's two applications. In the final paragraph, Applicant merely asserts a conclusory statement without articulating an argument. Further, the last sentence appears to be a mistaken cut-and-paste from a different application; clarification is requested.

The rejection stands as set forth above.

Conclusion

18. No claim is allowed.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to RUSSELL T BOGGS whose telephone number is (571)272-2805. The examiner can normally be reached on Monday - Friday, 0800 to 1830 Mtn.

Examiner interviews are available via telephone, in-person, and video conferencing using a USPTO supplied web-based collaboration tool. To schedule an interview, applicant is encouraged to use the USPTO Automated Interview Request (AIR) at http://www.uspto.gov/interviewpractice.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Amjad Abraham can be reached on 571-270-0708. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free). If you would like assistance from a USPTO Customer Service Representative or access to the automated information system, call 800-786-9199 (IN USA OR CANADA) or 571-272-1000.

/RUSSELL T BOGGS/
Examiner, Art Unit 1663

/CATHY KINGDON WORLEY/
Primary Examiner, Art Unit 1662

I hereby certify that this paper (along with any paper referred to as being attached or enclosed) is being transmitted via the Office electronic filing system in accordance with 37 CFR § 1.6(a)(4).

Dated: 4/23/18 Signature: Kirsten Book
(Kirsten Book)

Docket No.: 091876-0027 **PATENT**

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of: Willem Hendrik LINDHOUT, et al.	:	Customer Number: 23630
Application No.: 14/112,747	:	Confirmation No.: 5919
Filed: December 23, 2013	:	Art Unit: 1663
For: Hybrid Seed Potato Breeding	:	Examiner: Russell T. BOGGS

AMENDMENT IN RESPONSE TO FINAL OFFICE ACTION

Mail Stop - Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

INTRODUCTORY COMMENTS

In response to the non-final Office Action dated December 22, 2017, please amend the above-identified U.S. patent application as directed herein. Reconsideration and further examination of the present application is requested in light of the following

Amendments to the Specification begin on Page 2.

Amendments to the Claims begin on Page 11.

Remarks/Arguments begin on Page 21.

AMENDMENTS TO THE SPECIFICATION

Please replace Table 10 at pages 84 to 90 as follows:

class	trait name	locus symbol	*germplasm source*	marker/ Chromosomal position	encoded protein	GenBank acession number	literature references
Tuber morphological traits							
D	Red anthocyanin	*D (R/Rpw)*	*Solanum tuberosum L.*	Chrom. 3	dihydroflavonol 4-reductase (DFR)	AY289924.1	Van Eck et al., (1994); De Jong et al., (2003)
D	Blue or purple pigments	*P*	*Solanum tuberosum L.*	Chrom. 11	flavonoid 3',5'-hydroxylase (F3'5'H)	AY675561 recessive allele cv. Rideau AY675560 recessive allele clone USW5281.2 AY675558 recessive allele clone USW5281.2 AY675559 dominant allele Jung et al clone USW5281.2	Jung et al., (2005)
D	Pigmentation of tuber skin	*I*, (*D* in tetraploids)	*Solanum tuberosum L.*	Chrom. 10	Stan2 is an R2R3 MYB transcription factor	A Stan2 (777) cDNA isolated from W5281.2 (AY841129)	De Jong, (1987); Jung et al., (2009)
C	Pigmented tuber flesh colour	*Pf*	*Solanum tuberosum L.*	Chrom. 10	an allelic variant or paralog of *Stan2* R2R3 MYB transcription factor		De Jong, (1987); Zhan et al., (2009)
C	Yellow flesh colour	*Y-locus or BCH or Chy2*	*Solanum tuberosum L.*	Chrom. 3	beta-carotene hydroxylase 2	HM013963	Bonierbale et al., (1988); Brown et al., (2006)

C	Orange flesh colour	*Chy2 + zep*	*Solanum tuberosum L.*	Chrom. 2	*Zep* (on Chromosome 2) is involved in the conversion of zeaxanthin into antheraxanthin, and in the conversion of antheraxanthin into violaxanthin	HM013964 and HM013965	Wolters et al, (2010)
B	Tuber shape	*Ro*	*Solanum tuberosum L. CxE*	Chrom. 10, TG34 - TG63			Van Eck et al., 1994.
Processing or tuber quality traits							
D	Amylose / amylopectine	*GBSS*	*Solanum tuberosum L.*	Chrom. 8	granule-bound starch synthase	X58453.1	Van der Leij et al., (1991)
C	chipping quality cold sweetening	PAIN	*Solanum tuberosum L.*	Chrom. 3	beta-fructofuranosidase or potato acid invertase	X70368.1	Li et al., (2008).
D	Starch content and french fry quality	SPS	*S.tuberosum*	Chrom. 7	sucrose phosphate synthase (sps)	EU684230.1, X73477.1	Zrenner et al., (1995)
C	chipping quality cold sweetening	InvGE/GF	*Solanum tuberosum L.*	Chrom. 9	invertase	AY341425.1 AJ133765, HQ110081, HQ197977	Li et al., (2008).

D	chipping quality cold sweetening	Invertase inhibitor	*Solanum tuberosum L.*	Chrom. 12	Isoforms of an invertase inhibitor	AY864819 AY864820 FJ810205 FJ810206 FJ810207 FJ810208 FJ810209 AY864821 GU980593, GU980594 INH2b*B2 Cryptic? 158 1 Vacuolar GU980595	Brummel et al., (2011).
D	Black spot bruising	*PPO*	*Solanum tuberosum L.*	Chrom. 8	Polyphenoloxidase PPO	POT33 allele U22922.1POT32 allele U22921.1POT72 allele U22923.1	Thygesen et al., (1995)
D	Starch content and french fry quality	Sucrose synthase	*Solanum tuberosumL.*	Chrom. 9	sucrose synthase	AY205302	
D	phenolic antioxidants	*StMtf1M*	*Solanum tuberosum L.*		MYB transcription factor gene StMtf1M to activate the phenylpropanoid biosynthetic pathway	EU310399	Rommens et al., (2008a)
D	acrylamide levels	*StAs1* and *StAs2*	*Solanum tuberosum L.*		asparagine synthetase	CK278037.1 CK271149.1	Rommens et al., (2008b)
D	Starch phosphate content and frying quality	*PhL*	*Solanum tuberosum L.*		phosphorylase-L (PhL)	AF143202	Rommens et al., (2006)
D	Starch phosphate content and frying quality	*GWD*	*Solanum tuberosum L.*	Chrom. 5	R1 protein or synonymous with Glucan Water Dikinase	Y09533.1	Ritte et al., (2002); Rommens et al., (2006)

D	Resistance to high temperature inhibition of tuber formation	*FT*	*Solanum tuberosum L.*		Flowering locus T protein	GU223211.1	Fan et al., (2010)
Resistance to *Phytopthera infestans*							
D	Resistance to *Phytophthera infestans*	*R1*	*Solanum demissum*	Chrom. 5	R1	AF447489.1	Leonards-Schippers *et al.* (1994); Ballvora, et al., (2002)
D	Resistance to *Phytophthera infestans*	*R2*	*Solanum demissum*	Chrom. 4	R2	FJ536325.1	Lokossou et al., (2009)
D	Resistance to *Phytophthera infestans*	*R2-like*	*Solanum sp. AM-3778-16*	Chrom. 4	AM-3778-16 R2-like protein gene	FJ536323.1	Lokossou et al., (2009)
D	Resistance to *Phytophthera infestans*	*Rpi-hjt1.3, Rpi-hjt1.2, Rpi-hjt1.1*	*Solanum hjertingii*	Chrom. 4	Rpi-hjt1.3, Rpi-hjt1.2, Rpi-hjt1.1	Rpi-hjt1.3 GU563973.1 Rpi-hjt1.2 GU563972.1 Rpi-hjt1.1 GU563971.1	Champouret, N. (2010)
D	Resistance to *Phytophthera infestans*	*Rpi-abpt*	*Solanum bulbocastanum*	Chrom. 4	ABPT Rpi	FJ536324.1	Lokossou et al., (2009)
D	Resistance to *Phytophthera infestans*	*R3a*	*Solanum demissum*	Chrom. 11	R3a	AY849382	El-Kharbotly et al. (1994); Huang et al., (2004; 2005)
D	Resistance to *Phytophthera infestans*	*R3b*	*Solanum demissum*	Chrom. 11	R3b	HQ437670.1	El-Kharbotly et al. (1994); Huang et al., (2004)
B	Resistance to *Phytophthera infestans*	*Rpi1*	*Solanum pinnatisectum*	Chrom. 7	Rpi1		Kuhl et al. (2001)
D	Resistance to *Phytophthera infestans*	*Rpi-vnt1.1*	*Solanum venturii*	Chrom. 9	Rpi-vnt1.1	FJ423044.1	Foster et al., (2009); Pel et al., (2009)
D	Resistance to *Phytophthera infestans*	*Rpi-vnt1.2*	*Solanum venturii*	Chrom. 9	Rpi-vnt1.2	FJ423045.1	Foster et al., (2009)

D	Resistance to *Phytophthera infestans*	*Rpi-vnt1.3*	*Solanum venturii*	Chrom. 9	Rpi-vnt1.3	FJ423046.1	Foster et al., (2009)
D	Resistance to *Phytophthera infestans*	*Rpi-blb1 / Rb*	*Solanum bulbocastanum*	Chrom. 8	Rpi-blb1 / Rb	AY336128 / AY426259 /	Van der Vossen et al., (2003); Song et al., (2003)
D	Resistance to *Phytophthera infestans*	*Rpi-blb2*	*Solanum bulbocastanum*	Chrom. 6	Rpi-blb2	DQ122125.1	Lokossou et al., (2009)

D	Resistance to *Phytophthera infestans*	*Rpi-bt1*	*Solanum bulbocastanum*	Chrom. 8, between CD60 and TG261	Rpi-bt1	FJ188415.1	Oosumi et al., (2009)
B	Resistance to *Phytophthera infestans*	*R5*	*Solanum demissum*	Chrom. 11	R5		Huang S (2005)
B	Resistance to *Phytophthera infestans*	*R6*	*Solanum demissum*	Chrom. 11	R6		El-Kharbotly *et al.* (1996)
B	Resistance to *Phytophthera infestans*	*R7*	*Solanum demissum*	Chrom. 11	R7		El-Kharbotly et al. (1996)
A	Resistance to *Phytophthera infestans*	*R8*	*Solanum demissum*		R8		Black et al., (1953); Malcolmson et al., (1966).
B	Resistance to *Phytophthera infestans*	*R9*	*Solanum demissum*	Chrom. 11	R9		Malcolmson et al., (1966), Huang S (2005)
B	Resistance to *Phytophthera infestans*	*R10*	*Solanum demissum*	Chrom. 11	R10		Huang S (2005)
B	Resistance to *Phytophthera infestans*	*R11*	*Solanum demissum*	Chrom. 11	R11		Huang S (2005)
B	Resistance to *Phytophthera infestans*	*Rpi-ber1*	*Solanum berthaultii*	Chrom. 10, between CT214 and TG63	Rpi-ber1		Ewing et al. 2000; Rauscher et al., (2006) ; Park et al., (2009)
B	Resistance to *Phytophthera infestans*	*Rpi-mcd1*	*Solanum microdontum*	Chrom. 4			Tan et ., (2008); Tan et al., 2010)
Resistance to nematodes							

B	Resistance to *G. rostochiensis Ro1*	*H1*	*Solanum tuberosum subsp. andigena CPC 1673*	Chrom. 5, linked to CP113 and CD78			Pineda *et al.* (1993); Gebhardt *et al.* (1993)
B	Resistance to *G. pallida*	*H3 or $GpaIV_{adg}$*	*Solanum tuberosum subsp. Andigena*	Chrom. 4: Marker assay:BAC9_3_fwd_ CGCCAACTCTGCTTC ATACABAC9_3_rev_ TGCAGAGGTCAGTTG TTTGG. SNP markers were developed from BACs GB003E01 and GB080G22 submitted to GenBank as AC236732 and AC236733, respectively.			Bradshaw et al., (1998); Moloney et al., (2010)
D	Resistance to *G. rostochiensis* Ro1	*Gro1-4*	*Solanum spegazzinii*	Chrom. 7		AY196151	Paal, et al., (2004)
B	Resistance to Globodera rostochiensis Ro5-22 and G. pallida Pa2-D383	*Grp1*	*Solanum tuberosum* 78-3778 ***(AM)***	Chrom. 5			Rouppe van der Voort et al., (1998)

B	Resistance to *G. pallida* Pa2, Pa3	*Gpa*	*Solanum spegazzinii*	Chrom. 5			Kreike *et al.* 1994
C	Resistance to *G. pallida*	*Gpa2*	*Solanum tuberosum Subsp. Andigena Cpc 1673*	Chrom. 12, between GP34 and CT79		AF195939.1	Van der Vossen et al., (2000); Bakker et al., (2003).
B	Resistance to *G. pallida* Pa2, Pa3	*GpaXI* $_{tar}$ I	*Solanum tarijense*	Chrom. 11			Tan et al., (2009).
B	Resistance to *G. pallida* Pa2, Pa3	*Gpa5*	*3704-76 (JP)*	Chrom. 5: forward primer 5' ACACCACCTGTTTGA TAAAAAACT 3' with the reverse primer 5' GCCTTACTTCCCTGCT GAAG 3' resulted in haplotype c			Rouppe van der Voort *et al.* (2000); Sattarzadeh et al., (2006)
B	Resistance to G. pallida Pa3	*Gpa6*	*3704-76*	Chrom. 9			Rouppe van der Voort et al. (2000); Sattarzadeh et al., (2006)
B	Resistance to *M. chitwoodi*	R_{Mc1}	*Solanum bulbocastanum*	Chrom. 11			Brown *et al.* (1996); Rouppe van der Voort et al. (1999)
B	Resistance to *M. chitwoodi/M. fallax*	*RMc2*	*Solanum fendlerii*	Chrom. 11			Draaistra (2006)
B	Resistance to *M. chitwoodi/M. fallax*	*RMc1(hou)*	*Solanum hougassii*	Chrom. 11			Brown *et al.*, (2003)
A	Resistance to *M. hapla*	*RMh1*	*Solanum chacoense* BGRC18618				Draaistra (2006)
A	Resistance to *M. hapla*	*RMh2*	*Solanum chacoense* BGRC18618				Draaistra (2006)
A	Resistance to *M. hapla*	*RMh3*	*Solanum tarijense* BGRC 24717				Draaistra (2006)

Resistance to viruses							
C	Resistance to PVY	Ry_{adg}	*Solanum tuberosum subsp andigena*	Chrom. 11		Y15297.1; AJ300266.1	Hämäläinen *et al.* (1997); Sorri et al (1999); Kasai et al., (2000); Vidal et al., (2002)
B	Resistance to PVY	*Rysto*	*Solanum stoloniferum*	Chrom. 12			Song, et al., (2005; 2008)
D	Resistance to PVX	*Rx*	*Solanum tuberosum subsp andigena CPC 1673**	Chrom. 12		AJ011801.1	Ritter *et al.* (1991); Bendahmane et al. (1999); Kanyuka et al., (1999)
B	Resistance to (PVX)	*Nb*	*S. tuberosum cv* Pentland Ivory	Chrom. 5, between GP21 and TG432			De Jong *et al.* (1997); Marano et al., (2002)
B	Resistance to (PVS)	*Ns*	*Solanum tuberosum DW 83-3121*	Chrom. 8, near GP126, GP189 and CP16			Marczewski *et al.*, (2002)

AMENDMENTS TO THE CLAIMS

1. (Currently amended) A diploid, fertile, self-compatible and essentially homozygous *Solanum tuberosum* potato line comprising plants having an average tuber yield expressed in grams of fresh weight of at least 200 grams per plant, wherein said line in addition to said tuber yield comprises at least one, more preferably at least 2, 3, 4, 5 or 6 agronomically desirable traits or genes, wherein said self-compatibility originates from *Solanum chacoense* and is referred to as S-locus inhibitor (*Sli*) gene, [[as]] a copy of said gene is present in a plant of potato lines NCIMB accession number 41663, NCIMB accession number 41664, NCIMB accession number 41665, or NCIMB accession number 41765, representative seeds of said lines having been deposited with the NCIMB, Aberdeen, Scotland under breeders reference AGVD1, AGVD2, AGVD3, and AGVD17 respectively, and wherein said at least one agronomically desirable trait or gene ~~is selected from the group consisting of *S. bulbocastanum* BGRC 8005-derived~~ provides resistance to *Phytophthora infestans* infections. ~~resistance locus *Rpi-blb1*; *S. bulbocastanum* clone PT29/ BAC clone CB3A14-derived *Phytophthora infestans* resistance gene *Rb* (Genbank acc. No. AY303170); *S. bulbocastanum* clone ARD 1197-16-derived *Phytophthora infestans* resistance locus *Rpi-blb2* (Genbank acc. No. DQ122125); *S. bulbocastanum* clone Blb99-256-3-derived *Phytophthora infestans* resistance locus *Rpi-blb3*; *S. bulbocastanum/S. tuberosum* somatic hybrid J101-derived *Phytophthora infestans* resistance locus Rpi-bt1; *S. demissum* Black 3053-18-derived *Phytophthora infestans* resistance locus R5; *S. demissum* Black tetraploid clone MaR6-derived *Phytophthora infestans* resistance locus R6; *S. demissum* tetraploid clone MaR7-derived *Phytophthora infestans* resistance locus R7; *S. demissum* host 2424 a (5)-derived *Phytophthora infestans* resistance locus R8; *S. demissum* host 2573 (2)-derived *Phytophthora infestans* resistance locus R9; *S. demissum* Black 3681 ad (1)-derived *Phytophthora infestans* resistance locus R10; *S. demissum* Black 5008 ab (6) derived *Phytophthora infestans* resistance locus R11; *S. tuberosum* subsp. *andigena* CPC1673-derived *G. rostochiensis* Ro1 resistance locus H1; *S. tuberosum* subsp. *andigena* CPC2802-derived *G. pallida* resistance loci *H3* or *GpalVadg*; *S. tuberosum* AM78-3778-derived *G. rostochiensis* and *G. pallida* resistance locus *Grp1*;~~

S. spegazzinii BGRC 8218-15-derived *G. pallida* pathotypes Pa2 and Pa3 resistance locus *Gpa*; *S. tarijense* BGRC 24717-derived *G. pallida* pathotypes Pa2 and Pa3 resistance locus *GpaXI tar-1*; *S. tuberosum* 3704-76 (JP)-derived *G. pallida* pathotypes Pa2 and Pa3 resistance locus *Gpa5* and pathotype Pa3 resistant locus *Gpa6*; *S. bulbocastanum* PI 275187-derived *M. chitwoodi* resistance locus *Rmc1*; *S. fendlerii* BGRC 8083-derived *M. chitwoodi* and *M. fallax* resistance locus *Rmc2*; *S. hougassii* PI 161726-derived *M. chitwoodi* resistance locus *Rmc1(hou)*; *S. chacoense* BGRC 18618-derived *M. hapla* resistance loci *Rmh1* and *Rmh2*; *S. tarijense* BGRC 24717-derived *M. hapla* resistance locus *Rmh3*; *S. tuberosum* cv Pentland Ivory-derived *Potato Virus X* resistance locus; *S. tuberosum* DW 83-3121-derived *Potato Virus S* resistance locus *Ns*; *S. tuberosum* beta-carotene hydroxylase 2 gene *CHY2* (GenBank Acc No HM013963.1); *S. tuberosum* zeaxanthin epoxidase gene *ZEP* (ZEP-1 allele) (GenBank Acc No HM013964.1); *S. tuberosum* zeaxanthin epoxidase gene *ZEP* (ZEP-2 allele) (GenBank Acc No HM013965.1); *S. tuberosum* granule-bound starch synthase gene *GBSS* (GenBank Acc No X58453.1); *S. tuberosum* beta-fructofuranosidase gene *PAIN-1* (GenBank Acc No X70368.1); *S. tuberosum* acid invertase gene (GenBank Acc No AY341425.1); *S. tuberosum* sucrose-phosphate-synthase gene *sps* (GenBank Acc No EU684230.1); *S. tuberosum* invertase genes *invGE* and *invGF* (GenBank Acc No AJ133765.1); *S. tuberosum* apoplastic invertase gene *InvCD141* (GenBank Acc No HQ110081.1); *S. tuberosum* invertase gene *InvCD111* (GenBank Acc No HQ197977); *S. tuberosum* putative invertase inhibitor precursor gene *INH1* (INH1*A allele) (GenBank Acc No AY864819.1); *S. tuberosum* putative invertase inhibitor precursor gene *INH1* (INH1*B allele) (GenBank Acc No AY864820.1); *S. tuberosum* invertase inhibitor gene *INH2alpha* (INH2alpha*A1 allele) (GenBank Acc No FJ810205.1); *S. tuberosum* invertase inhibitor gene *INH2alpha* (INH2alpha*A2 allele) (GenBank Acc No FJ810206.1); *S. tuberosum* invertase inhibitor gene *INH2alpha* (INH2alpha*B allele) (GenBank Acc No FJ810207.1); *S. tuberosum* invertase inhibitor gene *INH2alpha* (INH2alpha*C allele) (GenBank Acc No FJ810208.1); S. tuberosum invertase inhibitor gene *INH2alpha* (INH2alpha*D allele) (GenBank Acc No FJ810209.1); *S. tuberosum* putative invertase inhibitor precursor gene *INH2beta* (INH2beta*A1 allele) (GenBank Acc No AY864821.1); *S. tuberosum* tuber polyphenol oxidase gene *PPO* (POT32 allele) (GenBank Acc No U22921.1); *S. tuberosum* tuber polyphenol oxidase gene *PPO* (POT33 allele) (GenBank Acc No U22922.1); *S. tuberosum* tuber polyphenol oxidase

gene *PPO* (POT72 allele) (GenBank Acc No U22923.1); *S. tuberosum* sucrose synthase gene *SuSy2* (GenBank Acc No AY205302.1); *S. tuberosum* chlorogenate inducer gene *Cai1* (GenBank Acc No EU310399.1); *S. tuberosum* asparagine synthetase expressed sequence tags EST724115 (GenBank Acc No. CK278037.1) and EST717227 (GenBank Acc No. CK271149.1); *S. tuberosum* starch phosphorylase gene *L* (GenBank Acc No AF143202.1); *S. tuberosum* glucan water dikinase gene *R1* (GenBank Acc No Y09533.1); *S. demissum* late blight resistance gene *R2* (GenBank Acc No FJ536325.1); *Solanum* sp. AM-3778-16 late blight resistance gene for *R2*-like protein (GenBank Acc No FJ536323.1); *S S. hjertingii* R2 late blight resistance gene *Rpi-hjt1.1* (GenBank Acc No. GU563971.1); *S. hjertingii* R2 late blight resistance gene *Rpi-hjt1.2* (GenBank Acc No. GU563972.1; *S. hjertingii* R2 late blight resistance gene *Rpi-hjt1.3* (GenBank Acc No. GU563973.1); *Solanum* sp. ABPT late blight resistance gene *Rpi* (GenBank Acc No FJ536324.1); *S. venturii* late blight resistance gene *Rpi-vnt1.1* (GenBank Acc No FJ423044.1); *S. venturii* late blight resistance gene *Rpi-vnt1.2* (GenBank Acc No FJ423045.1); *S. venturii* late blight resistance gene *Rpi-vnt1.3* (GenBank Acc No FJ423046.1); *S. bulbocastanum* putative disease resistant gene *RGA2* (GenBank Acc No AY336128.1); *S. bulbocastanum* blight resistance gene *RPI* (GenBank Acc No AY426259); *S. bulbocastanum* late blight resistance gene *Rpi-bt1* (GenBank Acc No FJ188415.1); *Solanum spegazzinii* nematode resistance gene *Gro1-4* (GenBank Acc No AY196151.1); *S. tuberosum* nematode resistance gene *Gpa2* (GenBank Acc No AF195939.1); *S. tuberosum* subsp. *andigena* potato Y and A potyvirus resistance gene *ADG2/2x(v-2)7a* (GenBank Acc No. Y15297.1); *S. tuberosum* subsp. *andigena* potato Y potyvirus resistance gene Y-1 (GenBank Acc No AJ300266.1) and splice variants C38 and C19 thereof; *S. tuberosum* dihydroflavonol 4-reductase gene (GenBank Acc No AY289924.1); *S. tuberosum* flavonoid 3',5'-hydroxylase (P locus) genes (GenBank Acc Nos. AY675561.1, AY675560.1, AY675558.1 and AY675559.1); *S. tuberosum* anthocyanin 1 gene *an1* (GenBank Acc No AY841129.1); *S. demissum* late blight resistance gene *R1* (GenBank Acc No AF447489.1); *S. demissum* potato late blight resistance gene *R3a* (GenBank Acc No AY849382); *S. tuberosum* potato virus X (PVX) resistance gene *rx* (GenBank Acc No AJ011801.1); *S. tuberosum* potato late blight resistance gene *R3b* (GenBank Acc No HQ437670.1); *S. tuberosum* flowering locus gene *T* (FT) (GenBank Acc No GU223211.1); *S. tuberosum* US-W5337.3 derived tuber shape gene locus *Ro*; *S. pinnatisectum* PI 253214-derived *Phytophthora*

~~*infestans* resistance locus *Rpi1*; *S. berthaultii* PI 473331-derived *Phytophthora infestans* resistance locus *Rpi-ber1*; and *S. microdontum* BGRC 18302(CGN21342)-derived *Phytophthora infestans* resistance locus *Rpi-mcd1*.~~

2. (Currently amended) A hybrid potato seed resulting from a cross between two essentially homozygous parental potato breeding lines, at least one of which is a plant of [[a]] the line according to claim 1.

3. (Previously presented) A hybrid potato plant grown from the seed of claim 2.

4. (Currently amended) A tetraploid plant produced by doubling the genome of a plant of [[a]] the line of claim 1.

5. (Currently amended) A method for producing a diploid, fertile, self-compatible and essentially homozygous *Solanum tuberosum* potato line comprising plants having an average tuber yield expressed in grams of fresh weight of at least 200 grams per plant, comprising:

(a) providing a first potato plant, which first potato plant is a plant of a first diploid, self-compatible, fertile and essentially homozygous potato line, wherein said self-compatibility originates from *Solanum chacoense* and is referred to as S-locus inhibitor (*Sli*) gene, [[as]] a copy of said gene is present in a plant of potato lines NCIMB accession number 41663, NCIMB accession number 41664, NCIMB accession number 41665, or NCIMB accession number 41765, representative seeds of said lines having been deposited with the NCIMB, Aberdeen, Scotland under breeders reference AGVD1, AGVD2, AGVD3, and AGVD17 respectively;

(b) providing a second potato plant, wherein said second potato plant is a plant of a diploid or tetraploid *Solanum tuberosum* potato line which may have any level of homozygosity, said line of said second potato plant comprising plants having an average tuber yield expressed in grams of fresh weight of at least 200 grams per plant;

(c) cross pollinating said first and second potato plant to provide seeds and collecting said seeds to thereby provide an offspring generation in the form of a hybrid offspring seed;

(d) growing said hybrid offspring seed into a population of hybrid offspring potato plant and selecting from said population plants producing tubers at a yield expressed in grams of fresh weight of at least 200 grams per plant, and

(e) selfing said hybrid offspring potato plant or backcrossing said hybrid offspring potato plant to a potato plant of said line of said first potato plant, for between 1 to 8 selfings or backcrosses, to thereby provide a diploid, self-compatible and essentially homozygous *Solanum tuberosum* potato breeding line comprising plants having an average tuber yield expressed in grams of fresh weight of at least 200 grams per plant;

(f) said method further comprising the step of introducing into a plant of said diploid, self-compatible and essentially homozygous potato breeding line obtained in step (e) at least one trait or gene ~~selected from the group consisting *S. bulbocastanum* BGRC 8005-derived~~ provides resistance to *Phytophthora infestans* infections. ~~resistance locus *Rpi-blb1*; *S. bulbocastanum* clone PT29/ BAC clone CB3A14-derived *Phytophthora infestans* resistance gene *Rb* (Genbank acc. No. AY303170); *S. bulbocastanum* clone ARD 1197-16-derived *Phytophthora infestans* resistance locus *Rpi-blb2* (Genbank acc. No. DQ122125); *S. bulbocastanum* clone Blb99-256-3-derived *Phytophthora infestans* resistance locus *Rpi-blb3*; *S. bulbocastanum/S. tuberosum* somatic hybrid J101-derived *Phytophthora infestans* resistance locus Rpi-bt1; *S. demissum* Black 3053-18-derived *Phytophthora infestans* resistance locus R5; *S. demissum* Black XD2-21-derived *Phytophthora infestans* resistance locus R6; *S. demissum* Black 2182-ef (7)-derived *Phytophthora infestans* resistance locus R7; *S. demissum* Black 2424-a (5)-derived *Phytophthora infestans* resistance locus R8; *S. demissum* Black 2573 (2)-derived *Phytophthora infestans* resistance locus R9; *S. demissum* Black 3681-ad (1)-derived *Phytophthora infestans* resistance locus R10; *S. demissum* Black 5008-ab (6)-derived *Phytophthora infestans* resistance locus R11; *S. tuberosum* subsp. *andigena* CPC1673-derived *G. rostochiensis* Ro1 resistance locus H1; *S. tuberosum* subsp. *andigena* CPC2802-derived *G. pallida* resistance loci *H3* or *GpaIVadg*; *S. tuberosum* AM78-3778-derived *G. rostochiensis* and *G. pallida* resistance locus *Grp1*;
S. spegazzinii BGRC 8218-15-derived *G. pallida* pathotypes Pa2 and Pa3 resistance locus *Gpa*; *S. tarijense* BGRC 24717-derived *G. pallida* pathotypes Pa2 and Pa3 resistance locus *GpaXI tar 1*; *S. tuberosum* 3704-76 (JP)-derived *G. pallida* pathotypes Pa2 and Pa3 resistance loci *Gpa5* and *Gpa6*;~~

S. bulbocastanum PI 275187-derived *M. chitwoodi* resistance locus *Rmc1*; *S. fendlerii* BGRC 8083-derived *M. chitwoodi* and *M. fallax* resistance locus *Rmc2*; *S. hougassii* PI 161726-derived *M. chitwoodi* resistance locus *Rmc1(hou)*; *S. chacoense* BGRC 18618-derived *M. hapla* resistance loci *Rmh1* and *Rmh2*; *S. tarijense* BGRC 24717-derived *M. hapla* resistance locus *Rmh3*; *S. tuberosum* cv Pentland Ivory-derived *Potato Virus X* resistance locus; *S. tuberosum* DW-83-3121-derived *Potato Virus S* resistance locus *Ns*; *S. tuberosum* beta-carotene hydroxylase 2 gene *CHY2* (GenBank Acc No HM013963.1); *S. tuberosum* zeaxanthin epoxidase gene *ZEP* (ZEP-1 allele) (GenBank Acc No HM013964.1); *S. tuberosum* zeaxanthin epoxidase gene *ZEP* (ZEP-2 allele) (GenBank Acc No HM013965.1); *S. tuberosum* granule-bound starch synthase gene *GBSS* (GenBank Acc No X58453.1); *S. tuberosum* beta-fructofuranosidase gene *PAIN-1* (GenBank Acc No X70368.1); *S. tuberosum* acid invertase gene *Inv* (GenBank Acc No AY341425.1); *S. tuberosum* sucrose-phosphate-synthase gene *sps* (GenBank Acc No EU684230.1); *S. tuberosum* invertase genes *invGE* and *invGF* (GenBank Acc No AJ133765.1); *S. tuberosum* apoplastic invertase gene *InvCD141* (GenBank Acc No HQ110081.1); *S. tuberosum* invertase gene *InvCD111* (GenBank Acc No HQ197977); *S. tuberosum* putative invertase inhibitor precursor gene *INH1* (INH1*A allele) (GenBank Acc No AY864819.1); *S. tuberosum* putative invertase inhibitor precursor gene *INH1* (INH1*B allele) (GenBank Acc No AY864820.1); *S. tuberosum* invertase inhibitor gene *INH2alpha* (INH2alpha*A1 allele) (GenBank Acc No FJ810205.1); *S. tuberosum* invertase inhibitor gene *INH2alpha* (INH2alpha*A2 allele) (GenBank Acc No FJ810206.1); *S. tuberosum* invertase inhibitor gene *INH2alpha* (INH2alpha*B allele) (GenBank Acc No FJ810207.1); *S. tuberosum* invertase inhibitor gene *INH2alpha* (INH2alpha*C allele) (GenBank Acc No FJ810208.1); *S. tuberosum* invertase inhibitor gene *INH2alpha* (INH2alpha*D allele) (GenBank Acc No FJ810209.1); *S. tuberosum* putative invertase inhibitor precursor gene *INH2beta* (INH2beta*A1 allele) (GenBank Acc No AY864821.1);

S. tuberosum tuber polyphenol oxidase gene *PPO* (POT32 allele) (GenBank Acc No U22921.1); *S. tuberosum* tuber polyphenol oxidase gene *PPO* (POT33 allele) (GenBank Acc No U22922.1); *S. tuberosum* tuber polyphenol oxidase gene *PPO* (POT72 allele) (GenBank Acc No U22923.1); *S. tuberosum* sucrose synthase gene *SuSy2* (GenBank Acc No AY205302.1); *S. tuberosum* chlorogenate inducer gene *Cai1* (GenBank Acc No EU310399.1); *S. tuberosum* asparagine

synthetase expressed sequence tags EST724115 (GenBank Acc No. CK278037.1) and EST717227 (GenBank Acc No. CK271149.1); *S. tuberosum* starch phosphorylase gene *L* (GenBank Acc No AF143202.1); *S. tuberosum* glucan water dikinase gene *R1* (GenBank Acc No Y09533.1); *S. demissum* late blight resistance gene *R2* (GenBank Acc No FJ536325.1); *Solanum* sp. AM-3778-16 blight resistance gene for *R2*-like protein (GenBank Acc No FJ536323.1); *S S. hjertingii* R2 late blight resistance gene *Rpi-hjt1.1* (GenBank Acc No. GU563971.1); *S. hjertingii* R2 late blight resistance gene *Rpi-hjt1.2* (GenBank Acc No. GU563972.1; *S. hjertingii* R2 late blight resistance gene *Rpi-hjt1.3* (GenBank Acc No. GU563973.1); *Solanum* sp. ABPT late blight resistance gene *Rpi* (GenBank Acc No FJ536324.1); *S. venturii* late blight resistance gene *Rpi-vnt1.1* (GenBank Acc No FJ423044.1); *S. venturii* late blight resistance gene *Rpi-vnt1.2* (GenBank Acc No FJ423045.1); *S. venturii* late blight resistance gene *Rpi-vnt1.3* (GenBank Acc No FJ423046.1); *S. bulbocastanum* putative disease resistant gene *RGA2* (GenBank Acc No AY336128.1); *S. bulbocastanum* blight resistance gene *RPI* (GenBank Acc No AY426259); *S. bulbocastanum* late blight resistance gene *Rpi-blb2* (GenBank Acc No DQ122125.1); *S. bulbocastanum* late blight resistance gene *Rpi-bt1* (GenBank Acc No FJ188415.1); *Solanum spegazzinii* nematode resistance gene *Gro1-4* (GenBank Acc No AY196151.1); *S. tuberosum* nematode resistance gene *Gpa2* (GenBank Acc No AF195939.1 *S. tuberosum* subsp. *andigena* potato Y and A potyvirus resistance gene *ADG2/2x(v-2)7a* (GenBank Acc No. Y15297.1); *S. tuberosum* subsp. *andigena* potato Y potyvirus resistance gene Y-1 (GenBank Acc No AJ300266.1) and splice variants C38 and C19 thereof; *S. tuberosum* dihydroflavonol 4-reductase gene (GenBank Acc No AY289924.1); *S. tuberosum* flavonoid 3',5'-hydroxylase (P locus) genes (GenBank Acc Nos. AY675561.1, AY675560.1, AY675558.1 and AY675559.1); *S. tuberosum* anthocyanin 1 gene *an1* (GenBank Acc No AY841129.1); *S. demissum* late blight resistance gene *R1* (GenBank Acc No AF447489.1); *S. demissum* potato late blight resistance gene *R3a* (GenBank Acc No AY849382); *S. tuberosum* potato virus X (PVX) resistance gene *rx* (GenBank Acc No AJ011801.1); *S. demissum* potato late blight resistance gene *R3b* (GenBank Acc No HQ437670.1); *S. tuberosum* flowering locus gene *T* (FT) (GenBank Acc No GU223211.1); *S. tuberosum* US-W5337.3 derived tuber shape gene locus *Ro*; *S. pinnatisectum* PI 253214 derived *Phytophthora infestans* resistance locus *Rpi1*; *S. berthaultii*

~~PI 473331-derived *Phytophthora infestans* resistance locus *Rpi-ber1*; and *S. microdontum* BGRC 18302(CGN21342)-derived *Phytophthora infestans* resistance locus *Rpi-mcd1*.~~

6. (Previously presented) The method of claim 5, wherein said first potato plant is a plant of potato line NCIMB accession number 41663, NCIMB accession number 41664, NCIMB accession number 41665, or NCIMB accession number 41765, representative seeds of said line having been deposited with the NCIMB, Aberdeen, Scotland under breeders reference AGVD1, AGVD2, AGVD3, and AGVD17 respectively.

7. (Previously presented) A diploid potato breeding line obtained by the method of claim 5.

8. (Currently amended) A method for producing a uniform hybrid potato seed, comprising:

(a) providing a first potato plant of [[a]] the line according to claim 1,

(b) providing a second potato plant, wherein said second potato plant is a plant of a diploid, fertile, self-compatible and essentially homozygous potato line, and

(c) cross pollinating said first and second potato plant and allowing one of either said first or second potato plant to produce berries with seeds and collecting said seeds from said berries to provide an offspring generation in the form of a hybrid offspring seed.

9. (Original) A seed produced by the method of claim 8.

10. (Original) A hybrid potato plant, or part thereof, produced by growing the seed of claim 9.

11. (Currently amended) A [[P]]pollen or an ovule of the plant of claim 10.

12. (Original) A tuber from the plant of claim 10.

13. (Original) A potato plant having all of the physiological and morphological characteristics of the plant of claim 10.

14. (Previously presented) A tissue culture of the plant of claim 10.

15. (Currently amended) A potato plant regenerated from the tissue culture of claim 14, wherein said regenerated potato plant has all the physiological and morphological characteristics of [[a]] the potato plant of claim 10.

16. (Original) A method of producing a hybrid potato plant, said method comprising producing a seed according to the method of claim 8 and growing said seed into a potato plant.

17. (Original) A potato plant produced by the method of claim 16, or a tuber thereof.

18. (Previously Presented) A set of at least 2 intercrossable diploid, fertile, self-compatible and essentially homozygous potato lines according to claim 1.

19. (Previously presented) An elite diploid potato breeding line obtained by the method of claim 5.

20. (New) The diploid, fertile, self-compatible and essentially homozygous *Solanum tuberosum* potato line according to claim 1, wherein said at least one additional trait or gene that provides resistance to *Phytophthora infestans* infections is selected from the group consisting of *Solanum demissum* gene *R1* on chromosome 5; *S. demissum* gene *R2* on chromosome 4; *Solanum sp.* AM-3778-16 gene *R2*-like on chromosome 4; *S. bulbocastanum* gene *Rpi-blb1/Rb* on chromosome 8; *S. bulbocastanum* gene *Rpi-blb2* on chromosome 6; *S. bulbocastanum* gene *Rpi-bt1* on chromosome 8; *S. demissum* gene *R5* on chromosome 11; *S. demissum* gene *R6* on chromosome 11; *S. demissum* gene *R7* on chromosome 11; *S. demissum* gene *R9* on chromosome 11; *S. demissum* gene *R10* on chromosome 11; *S. demissum* gene *R11* on chromosome 11; *S. hjertingii* genes *Rpi-hjt1.1*, *Rpi-hjt1.2* and *Rpi-hjt1.3* on chromosome 4; *S. bulbocastanum Rpi-abpt* gene on chromosome 4; *S. venturii* genes *Rpi-vnt1.1*, *Rpi-vnt1.2*, and *Rpi-vnt1.3* on chromosome 9; *S. demissum* gene *R3a* on

chromosome 11; *S. demissum* potato gene *R3b* on chromosome 11; *S. pinnatisectum* gene *Rpi1* on chromosome 7; *S. berthaultii* gene *Rpi-ber1* on chromosome 10; and *S. microdontum* gene *Rpi-mcd1* on chromosome 4.

21. (New) The diploid, fertile, self-compatible and essentially homozygous *Solanum tuberosum* potato line according to claim 1, wherein said potato line is a progeny line of potato line IVP97-079-9, IVPAA-096-18 and/or SH83-92-488, which lines are available from the Laboratory for Plant Breeding, Wageningen University.

22. (New) The diploid, fertile, self-compatible and essentially homozygous *Solanum tuberosum* potato line according to claim 1, wherein said potato line is a progeny line of potato line NCIMB 41663, NCIMB 41664, NCIMB 41665, or NCIMB 41765, representative seeds of said NCIMB lines having been deposited with the NCIMB, Aberdeen, Scotland under breeders reference AGVD1, AGVD2, AGVD3, and AGVD17 respectively.

23. (New) The method according to claim 5, wherein the at least one trait or gene that is introduced into the plant of said diploid, self-compatible and essentially homozygous potato breeding line obtained in step (e) is selected from the group consisting of *Solanum demissum* gene *R1* on chromosome 5; *S. demissum* gene *R2* on chromosome 4; *Solanum sp.* AM-3778-16 gene *R2*-like on chromosome 4; *S. bulbocastanum* gene *Rpi-blb1/Rb* on chromosome 8; *S. bulbocastanum* gene *Rpi-blb2* on chromosome 6; *S. bulbocastanum* gene *Rpi-bt1* on chromosome 8; *S. demissum* gene *R5* on chromosome 11; *S. demissum* gene *R6* on chromosome 11; *S. demissum* gene *R7* on chromosome 11; *S. demissum* gene *R9* on chromosome 11; *S. demissum* gene *R10* on chromosome 11; *S. demissum* gene *R11* on chromosome 11; *S. hjertingii* genes *Rpi-hjt1.1*, *Rpi-hjt1.2* and *Rpi-hjt1.3* on chromosome 4; *S. bulbocastanum* Rpi-abpt gene on chromosome 4; *S. venturii* genes *Rpi-vnt1.1*, *Rpi-vnt1.2*, and *Rpi-vnt1.3* on chromosome 9; *S. demissum* gene *R3a* on chromosome 11; *S. demissum* potato gene *R3b* on chromosome 11; *S. pinnatisectum* gene *Rpi1* on chromosome 7; *S. berthaultii* gene *Rpi-ber1* on chromosome 10; and *S. microdontum* gene *Rpi-mcd1* on chromosome 4.

REMARKS/ARGUMENTS

Status of Claims

Claims 1-19 were pending in this application. Claims 1, 2, 4, 5, 8, 11 and 15 have been amended. New claims 20-23 are added.

Basis for this amendment is present throughout the application as filed. For example, support for the amendment to claims 1 and 5 can be found in the specification at page 6, lines 21-28. Support for new claims 20 and 23 can be found in the specification in Table 10 at pages 86-87. Support for new claim 21 can be found in the specification in Table 6 at page 59, and at page 49, lines 1-3. Support for new claim 22 can be found in the specification at page 34, line 29 to page 35, line 23.

Claims 2, 4, 8 and 15 have been amended to provide correct antecedent basis. Claim 11 is amended to correct certain informality. No new matter is believed to be added herein.

Specification

The Examiner has objected to the specification for listing non-patent literature in Table 10 on pages 84-90. Several of the claim terms in claims 1 and 5 stand rejected under 35 U.S.C. §112 (b) because they lack an art-accepted meaning.

In order to expedite prosecution of this application, Applicant has replaced Table 10 to that which was originally filed on December 23, 2013. The *Solanum tuberosum* potato lines, as claimed in amended claims 1 and 5, comprise at least one agronomically desirable trait or gene that provides resistance to *Phytophthora infestans* infections. Therefore, the claims do not incorporate all genes and all disease resistance traits listed in Table 10. The meaning and scope of the claimed features are therefore clear.

In addition, the traits or genes that provide resistance to *Phytophthora infestans* infections are recited in new claims 20 and 23 and possess an art-accepted meaning. For example, the *Solanum demissum* gene is known in the art for more than 60 years (*see* Black et al., Euphytica 1953, 2, 173-178 and Malcolmson and Black, Euphytica, 1966, 15, 199–203, cited in IDS filed on

June 16, 2017). These genes have been used in crosses that were reported in numerous scientific publications.

The nomenclature of late blight genes is as follows: R genes from *S. demissum* are named R1 till R11. Late blight R genes from other species are abbreviated as Rpi (resistance against *P. infestans*) followed by an abbreviation of the species name (blb from *S. bulbocastanum*, vnt from *Solanum venturii*, etc.). The subsequent number refers to the order of cloning of a particular R gene from that wild species.

Reconsideration and withdrawal of objection are respectfully requested.

Claim Objections

Claims 1, 2, 4, 5, 8, and 15 are objected to because of informalities.

A. Claims 1 and 5 are objected to because the difference between colons and semicolons are not easily distinguishable.

Applicant confirms that the claim amendments filed on June 15, 2017 used semicolons in claims 1 and 5. Amendments filed concurrently herewith are presented in a form having sufficient clarity and contrast to permit direct reproduction and are therefore in accordance with 37 CFR 1.52(a)(v).

B. Claims 2, 4, 8, and 15 are objected to for incorrect antecedent basis.

Claims 2, 4 and 8 are amended to recite "the line" and claim 15 is amended to recite "the potato plant of claim 10."

Reconsideration and withdrawal of objection are respectfully requested.

Improper Markush Group

Claims 1-19 are rejected on the judicially-created basis that they contain an improper Markush grouping of alternatives.

Claim 1 and 5 are amended and no longer contain a Markush grouping.

Reconsideration and withdrawal of objection are respectfully requested.

Rejections under 35 U.S.C. § 112 (b), second paragraph

Claims 1-19 are rejected under 35 U.S.C. § 112 (b), second paragraph, as being indefinite, for failing to particularly point out and distinctly claim the subject matter which Applicant regards as the invention.

According to the Examiner, claims 1 and 5 are indefinite in their recitation of numerous terms, for example "*S. bulbocastanum* BGRC 8005-derived *Phytophthora infestans* resistance locus *Rpi-blb1*; *S. bulbocastanum* clone PT29/ BAC clone CB3A14-derived *Phytophthora infestans* resistance gene *Rb* (Genbank acc. No. AY303170)," as the terms lack general art-accepted meanings.

Claims 1 and 5 are amended and no longer recite the terms that were rejected by the Examiner.a

Reconsideration and withdrawal of rejection are respectfully requested.

Rejections under 35 U.S.C. § 112(a), written description

Claims 1-5 and 7-19 are rejected under 35 U.S.C. 112(a) or 35 U.S.C. 112 (pre-AIA), first paragraph, as failing to comply with the written description requirement.

According to the Examiner, the claims are broadly drawn to any diploid, fertile, self-compatible and essentially homozygous *Solanum tuberosum* potato line and are not limited to the self-compatibility genes and/or potato lines described by the deposits.

Applicant respectfully disagrees. In order to expedite prosecution, the claims have been amended to recite a diploid, fertile, self-compatible and essentially homozygous *Solanum tuberosum* potato line. The self-compatibility originates from *S. chacoense* and is referred to as S-locus inhibitor (*Sli*) gene. This gene is present in the plants of the potato lines, the representative seeds of which have been deposited in accordance with the Budapest Treaty at the NCIMB, Aberdeen, Scotland, under accession numbers 41663, 41664, 41665, and 41765 respectively.

It is therefore submitted that the claims encompass the genus of self-compatibility that is present in the deposited lines.

Reconsideration and withdrawal of rejection are respectfully requested.

Rejections under 35 U.S.C. § 112(a), improper incorporation by reference

Claims 1-5 and 7-19 are rejected under 35 U.S.C. 112(a) or 35 U.S.C. 112 (pre-AIA), first paragraph, as failing to comply with the written description requirement because claims 1 and 5 make reference to database accession numbers at numerous places.

Claims 1 and 5 are amended to delete references to database accession numbers.

Reconsideration and withdrawal of rejection are respectfully requested.

Rejections under 35 U.S.C. § 112(a), new matter

Claims 1-19 are rejected under 35 U.S.C. 112(a) or 35 U.S.C. 112 (pre-AIA), first paragraph, as failing to comply with the written description requirement because the amendments to claims 1 and 5 contain new matter with regard to recitation of certain terms.

The terms "BGRC 8005", "PT29", "Genbank acc. No. AY303170", "1197" and "INH1*A or INH1*B" have been deleted from claims 1 and 5.

Reconsideration and withdrawal of rejection are respectfully requested.

Rejections under 35 U.S.C. § 112(a), enablement re: deposits

Claims 1-19 are rejected under 35 U.S.C. 112(a) or 35 U.S.C. 112 (pre-AIA), first paragraph, as failing to comply with the enablement requirement.

The Examiner acknowledges that the Applicant has deposited the seeds as per the terms of the Budapest Treaty and also notes that a Statement was made by the Attorney on September 14, 2016 (*see* pages 22 and 23 of the Office Action dated December 22, 2017). However, according to the Examiner, the Applicant has not affirmed that the deposits recited in claim 1 will be irrevocably and without restriction be released to the public upon the issuance of a patent.

Applicant has concurrently filed herewith a Statement by an Attorney of record over her signature and registration number, affirming that the deposits recited in claim 1 will be irrevocably and without restriction be released to the public upon the issuance of a patent. Applicant has therefore perfected the deposit.

Reconsideration and withdrawal of rejection are respectfully requested.

Rejections under 35 U.S.C. § 112(a), enablement re: "A, B, C, and D" listings

Claims 1-19 are rejected under 35 U.S.C. 112(a) or 35 U.S.C. 112 (pre-AIA), first paragraph, as failing to comply with the enablement requirement.

Applicant has concurrently filed herewith a Statement by an Attorney of record over her signature and registration number, affirming that the deposits recited in claim 1 will be irrevocably and without restriction be released to the public upon the issuance of a patent.

Applicant has therefore provided evidence regarding the deposit of the representative seeds of the claimed *Solanum tuberosum* potato line and that the plant is readily available to the public. Therefore, the limitations in claims 1 and 5 are sufficiently enabled.

Reconsideration and withdrawal of rejection are respectfully requested.

Rejections under 35 U.S.C. § 112(a), re: Ex parte Jackson

Claims 1-5 and 7-19 are rejected under 35 U.S.C. 112(a) or 35 U.S.C. 112 (pre-AIA), first paragraph, because the specification while enabling for the deposited lines, does not reasonably provide enablement for forms of self-compatibility.

Claims have been amended and are drawn to the *Solanum tuberosum* potato line that is drawn solely to the deposits. The self-compatibility originates from *S. chacoense* and is referred to as S-locus inhibitor (*Sli*) gene. This gene is present in a plant of the potato lines deposited under NCIMB accession numbers 41663, 41664, 41665, and 41765. Therefore no degree of experimentation is involved in locating the genes from the deposited lines.

Reconsideration and withdrawal of rejection are respectfully requested.

Double Patenting

Claims 1-19 are provisionally rejected on the ground of nonstatutory obviousness-type double patenting as being unpatentable over claims 3 and 9 of copending Application No. 13/504,156.

Applicant respectfully disagrees.

Claims 3 and 9 of the copending Application No. 13/504,156 are drawn to a potato plant and methods of producing a diploid, fertile and self-compatible *Solanum tuberosum* potato line comprising a plant having a tuber yield expressed in grams of fresh weight of at least 200 grams; wherein at least 50% of genomic loci in said plant are homozygous, wherein said self-compatibility originating from *Solanum chacoense* as present in a plant of potato lines NCIMB accession number 41663, NCIMB accession number 41664, NCIMB accession number 41665, or NCIMB accession number 41765, representative seeds of said lines having been deposited with the NCIMB, Aberdeen, Scotland under breeders reference AGVD1, AGVD2, AGVD3, and AGVD17 respectively, and wherein at least 25% of the plants of said line produce seed-carrying berries, each carrying at least 5 seeds.

Amended claims 1 and 5 of the instant application are drawn to a diploid, fertile, self-compatible and essentially homozygous *Solanum tuberosum* potato line comprising an average tuber yield of at least 200 grams per plant, wherein said line comprises at least one or more agronomically desirable traits or genes and wherein said self-compatibility originating from *Solanum chacoense* and is referred to as S-locus inhibitor (*Sli*) gene, a copy of said gene is present in a plant of potato lines NCIMB accession number 41663, NCIMB accession number 41664, NCIMB accession number 41665, or NCIMB accession number 41765, representative seeds of said lines having been deposited with the NCIMB, Aberdeen, Scotland under breeders reference AGVD1, AGVD2, AGVD3, and AGVD17 respectively and wherein said at least one agronomically desirable traits or gene provides resistance to *Phytophthora infestans* infections.

An obviousness-type double patenting rejection is appropriate only when the claims of one application are obvious variants of the claims in another application or patent. The pending claims are not obvious variants because there is nothing in claims 3 and 9 of co-pending Application No. 13/504,156 that fairly suggests the subject matter of amended claim 1.

Reconsideration and withdrawal of the rejection are respectfully requested.

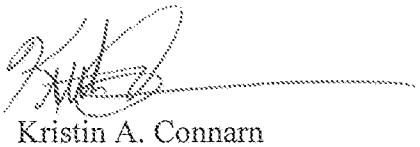
CONCLUSION

Should the Examiner be of the view that an interview would expedite consideration of the application, request is made that the Examiner telephone the Applicant's attorney at (617) 535 4453 in order that any outstanding issues be resolved.

Applicant has included the fee for one-month extension of time fee. The Director is hereby authorized to charge any deficiency in the fees filed, asserted to be filed, or which should have been filed herewith (or with any paper hereafter filed in this application by this firm) to our Deposit Account No. 50-1133, under Order No. 091876-0027 from which the undersigned is authorized to draw.

Respectfully submitted,

McDERMOTT WILL & EMERY LLP



Kristin A. Connarn
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Date: April 23, 2015

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
14/112,747	12/23/2013	Willem Hendrik Lindhout	091876-0027	5919

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McDermott Will & Emery
The McDermott Building
500 North Capitol Street, N.W.
Washington, DISTRICT OF COLUMBIA 20001
UNITED STATES OF AMERICA

EXAMINER
BOGGS, RUSSELL T

ART UNIT	PAPER NUMBER
1663	

NOTIFICATION DATE	DELIVERY MODE
08/07/2018	ELECTRONIC

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

ipdocketboston@mwe.com
mweipdocket@mwe.com

Office Action Summary	Application No. 14/112,747	Applicant(s) Lindhout et al.	
	Examiner RUSSELL T BOGGS	Art Unit 1663	AIA Status No

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTHS FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☑ Responsive to communication(s) filed on 23 April 2018.

☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on _____.

2a)☑ This action is **FINAL.** 2b) ☐ This action is non-final.

3)☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on _____; the restriction requirement and election have been incorporated into this action.

4)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims*

5) ☑ Claim(s) 1-23 is/are pending in the application.

5a) Of the above claim(s) _____ is/are withdrawn from consideration.

6) ☐ Claim(s) _____ is/are allowed.

7) ☑ Claim(s) 1-23 is/are rejected.

8) ☐ Claim(s) _____ is/are objected to.

9) ☐ Claim(s) _____ are subject to restriction and/or election requirement

* If any claims have been determined allowable, you may be eligible to benefit from the **Patent Prosecution Highway** program at a participating intellectual property office for the corresponding application. For more information, please see http://www.uspto.gov/patents/init_events/pph/index.jsp or send an inquiry to **PPHfeedback@uspto.gov.**

Application Papers

10)☑ The specification is objected to by the Examiner.

11)☐ The drawing(s) filed on _____ is/are: a)☐ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

Priority under 35 U.S.C. § 119

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

Certified copies:

a)☐ All b)☐ Some** c)☐ None of the:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

** See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☑ Notice of References Cited (PTO-892)

2) ☑ Information Disclosure Statement(s) (PTO/SB/08a and/or PTO/SB/08b) Paper No(s)/Mail Date _____.

3) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date _____.

4) ☐ Other: _____.

DETAILED ACTION

Notice of Pre-AIA or AIA Status

The present application is being examined under the pre-AIA first-to-invent provisions.

Status

1. Claims 1-18, filed 18 October 2013, were examined and rejected in the Office action mailed 14 March 2016. Applicant responded on 14 September 2016. Claims 1-19 were examined and rejected on 15 December 2016. Applicant filed an RCE and responded to the final Office action on 15 June 2017. Claims 1-19 were examined and rejected on 22 December 2017. Applicant responded on 23 April 2018, adding claims 20-23

Claims 1-23 are examined herein.

Withdrawal of Objections and Rejections

3. The objections to claims 1-2, 4-5, 8 and 15 are withdrawn in view of Applicant's amendments to the claims and through the use of a clear font in accordance with 37 CFR 1.52(a)(v).

4. The rejection of claims 1-19 for containing an improper Markush group in claims 1 and 5 is withdrawn in view of Applicant's amendments to the claims.

5. The rejection of claims 1-19 under the written description of 35 USC 112(a) requirement because the amendments to claims 1 and 5 contained new matter is withdrawn in view of Applicant's amendments to the claims.

6. The rejection of claims 1-19 under 35 U.S.C. 112(a) or 35 U.S.C. 112 (pre-AIA), first paragraph, as failing to comply with the enablement requirement regarding the deposited potato seeds and/or lines as set forth on pages 20-23 of the Office action mailed on 22 December 2017 is withdrawn in view of Applicant's statements on the record.

7. The double patenting rejection of claims 1-19 in view of application 13/504,156 is withdrawn.

Specification

8. The disclosure is objected to because of the following informalities.

The specification lists non-patent literature in Table 10. Several of the claim limitations in claims 20 and 23 are rejected under 35 USC 112(b) as being indefinite because they lack an art-accepted meaning. Applicant apparently intended to define the claim terms by reference to this table. Thus it is the Office's interpretation that it is Applicant's clear intent to incorporate the essential material cited by reference to the non-patent literature into the claims. The meaning and scope of the claim limitations can only be understood by reference to listed references, however, such an incorporation is improper.

The incorporation of essential material into specification by reference to an unpublished U.S. application, foreign application or patent, to a publication, or to an online database is improper. Applicant is required to amend the specification, if possible, to include the material incorporated by reference, if the material is relied upon to overcome any objection, rejection, or other requirement imposed by the Office. The

amendment must be accompanied by a statement executed by the applicant, or a practitioner representing the applicant, stating that the material being inserted is the material previously incorporated by reference and that the amendment contains no new matter. 37 CFR 1.57(f).

Applicant's Argument and Response

Applicant argued that replacing the table to the original version should obviate the objection. Response, pp. 21-22.

Applicant, however, is mistaken, for the reasons stated above.

Amendments of claims 20 and 23 to remove all reference to material only defined by reference to Table 10 would obviate this objection since then it would no longer be essential material.

Claim Rejections - 35 USC § 112(b)

The following is a quotation of 35 U.S.C. 112(b):

> (B) CONCLUSION.—The specification shall conclude with one or more claims particularly pointing out and distinctly claiming the subject matter which the inventor or a joint inventor regards as the invention.

The following is a quotation of 35 U.S.C. 112 (pre-AIA), second paragraph:

> The specification shall conclude with one or more claims particularly pointing out and distinctly claiming the subject matter which the applicant regards as his invention.

9. Claims 1-23 are rejected under 35 U.S.C. 112(b) or 35 U.S.C. 112 (pre-AIA), second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which the inventor or a joint inventor, or for pre-AIA the applicant regards as the invention.

Claim 1 is rejected because it recites the limitation "said gene" in line 2. The antecedent basis for this limitation in the claim is ambiguous because this limitation may refer to one of up to 7 different genes that are referenced earlier in the claim.

Claim 1 is rejected because it recites the limitation "said at least one agronomically desirable trait or gene" in line 10. The antecedent basis for this limitation in the claim is ambiguous because this limitation may refer to one of up to 6 different agronomically desirable traits or genes that are referenced earlier in the claim.

Claim 21 is rejected because it is drawn to a progeny line of a line available at a specific laboratory at Wageningen University. Among other reasons, this is not an internationally recognized depository. Additionally, there is no guarantee that the lines will be available through the lifetime of the patent. Furthermore, the term “Laboratory for Plant Breeding” could arbitrarily change to something different during the lifetime of a patent. Thus, one's ability to determine the metes and bounds of the claim would be impaired. See *In re Hammack*, 427 F .2d 1378, 1382; 166 USPQ 204, 208 (CCPA 1970).

Claims 20 and 23 are rejected because they recite numerous limitations in which genes, for example, are identified by a variety of terms where the meaning of the terms are indefinite. For example, the limitations in claims 20 and 23 beginning on line 3 of each recite, for example, various “R” genes located on a variety of chromosomes.

These terms lack general art-accepted meanings and Applicant does not explicitly define the terms in the specification, for example, by reference to the sequence listing or by reference to a specific location in a deposited cell line. Applicant provides Table 10 on pages 84 to 90 of the specification which cites references from non-patent literature or online databases, however, the incorporation of essential material in the specification by reference to an unpublished U.S. application, foreign application or patent, to a publication, or to an online database is improper. 37 C.F.R. 1.57(c).

Because of the indefiniteness of these terms , the metes and bounds of the claims cannot be determined. Furthermore, the meaning of these terms could arbitrarily change to designate something different during the lifetime of a patent. Thus, one's ability to determine the metes and bounds of the claim would be impaired. See *In re Hammack*, 427 F .2d 1378, 1382; 166 USPQ 204, 208 (CCPA 1970).

Dependent claims are included in this rejection because none provide limitations obviating this rejection.

Claim Rejections - 35 USC § 112(a), written description

The following is a quotation of 35 U.S.C. 112(a):

> (a) IN GENERAL.—The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same, and shall set forth the best mode contemplated by the inventor or joint inventor of carrying out the invention.

The following is a quotation of 35 U.S.C. 112 (pre-AIA), first paragraph:

> The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the

art to which it pertains, or with which it is most nearly connected, to make and use the same and shall set forth the best mode contemplated by the inventor of carrying out his invention.

10. Claims 1-5 and 7-23 are rejected under 35 U.S.C. 112(a) or 35 U.S.C. 112 (pre-AIA), first paragraph, as failing to comply with the written description requirement. The claim(s) contains subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor or a joint inventor, or for pre-AIA the inventor(s), at the time the application was filed, had possession of the claimed invention.

Due to Applicant's amendments to the claims, the rejection is modified from the rejection set forth in the Office action mailed 22 December 2017 as applied to the above-referenced claims. Applicant traverses the rejection in the paper filed 23 April 2018. Applicant's arguments have been fully considered but are not persuasive.

Claim 1 is drawn to a diploid, fertile, self-compatible and essentially homozygous *Solanum tuberosum* potato line having an average tuber yield expressed in grams of fresh weight of at least 200 grams per plant; wherein said line additionally comprises 1-6 agronomically desirable traits or genes where the trait provides resistance to *P. infestans* and wherein the self-compatibility originates from *S. chacoense* and is present in a plant of potato lines NCIMB accession number 41663, NCIMB accession number 41664, NCIMB accession number 41665, or NCIMB accession number 41765, further where representative samples were deposited with the NCIMB under breeders reference AGVD1, AGVD2, AGVD3, and AGVD17.

Claims 2-4 depending from claim 1 read on a hybrid potato plant or seed produced from a cross between two essentially homozygous parental breeding lines and a potato plant(s) produced therefrom. Claim 8 reads on a method using a plant

from claim 1. Claims 9-13 read on plants and plant parts produced by the method of claim 8. Claim 14 reads on a tissue culture produced by the plant of claim 10 and claim 15 reads on a plant produced from the tissue culture of claim 14. Claim 16 reads on a method using a seed produced according to claim 8 to produce a plant; claim 17 reads on a plant or tuber produced according to claim 16. Claim 18 reads on a set of at least 2 intercrossable diploid, fertile, self-compatible and essentially homozygous *S. tuberosum* potato lines according to claim 1.

New claims 20-22 depend from claim 1. Dependent claim 20 recites various embodiments of genes encoding *P. infestans* resistance. Dependent claim 21 recites that the potato line used is a progeny of a line available at a laboratory at Wageningen University. Dependent claim 22 recites that the potato line used is a progeny of a deposited line.

Claim 5 is a method claim drawn to producing a diploid, fertile, self-compatible and essentially homozygous *Solanum tuberosum* potato line starting with a first potato plant of a first diploid self-compatible, fertile and essentially homozygous potato line wherein the self-compatibility originates from *S. chacoense* and is present in a plant of potato lines NCIMB accession number 41663, NCIMB accession number 41664, NCIMB accession number 41665, or NCIMB accession number 41765, further where representative samples were deposited with the NCIMB under breeders reference AGVD1, AGVD2, AGVD3, and AGVD17 and crossing it to a second potato plant where the second potato plant has an average tuber yield expressed in grams of fresh weight of at least 200 grams per plant and then selfing or backcrossing to provide a diploid, self-compatible and essentially homozygous *S. tuberosum* potato breeding line

comprising plants having an average tuber yield of at least 200 grams per plant, and then introducing into a plant of said line at least one trait or gene where the gene or trait provides resistance to *P. infestans*.

Dependent claim 6 recites specific deposited cell lines and is not rejected under the written description requirement; claim 7 encompasses a diploid potato breeding line obtained by the method of claim 5. Claim 19 reads on an elite diploid potato breeding line obtained by the method of claim 5. New claim 23 depends from claim 5 and recites various embodiments of genes encoding *P. infestans* resistance.

The claimed genera, in particular, include those set forth in the following exemplary claims:

claim 1 – any "diploid, fertile, self-compatible and essentially homozygous *Solanum tuberosum* potato line" wherein it has a tuber yield of at least 200 g per plant and wherein the self-compatibility gene originates from *S. chacoense* and is present in a plant of potato lines NCIMB accession number 41663, NCIMB accession number 41664, NCIMB accession number 41665, or NCIMB accession number 41765;

claim 5 – any "diploid, fertile, self-compatible and essentially homozygous *Solanum tuberosum* potato line" wherein it has a tuber yield of at least 200 g per plant and wherein the self-compatibility gene originates from *S. chacoense* and is present in a plant of potato lines NCIMB accession number 41663, NCIMB accession number 41664, NCIMB accession number 41665, or NCIMB accession number 41765 as a starting material in part (a) to produce a "diploid, fertile, self-compatible and essentially homozygous *Solanum tuberosum* potato line" with an added trait or gene, wherein claim

5 is a method claim but several dependent claims read on compositions produced by the method.

The above claims, however, are not limited to potato lines described by the deposits.

Additionally, claim 13 represents a broad genus. Claim 13 requires all the physiological and morphological characteristics of the plant of claim 10, which is a plant produced by the method of claim 8, which starts with the plant of claim 1. Claim 1 recites only a handful of physiological and morphological characteristics – arguably just the tuber weight, and because claim 8 is a cross with another plant, the physiological and morphological characteristics which will be passed to any given offspring is uncertain and thus the plants produced by the method of claim 8 constitute a large genus.

Furthermore, claims 21 and 22 are drawn to any progeny line of either a line available at a laboratory or the deposited lines. First, claim 21 is rejected under 35 USC 112(b) as being indefinite. Second, claim 22 is unlimited with respect to the generation and thus may bear little resemblance to the deposited lines, which, as noted above, have only minimal description.

To claim a genus under the written description requirement, Applicant is required to describe a representative number of species to reflect the variation within the genus or structures sufficient to define the genus. The factors to be considered include disclosure of complete or partial structure, physical and/or chemical properties, functional characteristics, structure/function correlation, methods of making the claimed product, or any combination thereof.

In the specification, Applicant describes obtaining the IVP07-1001/4 line from publically available germplasm and plants that contain the *Sli* gene and using these plants to breed AGVD1, AGVD2, and AGVD3. Spec., pp. 46-47.

Applicant describes the deposited lines by breeders reference: AGVD1 (NCIMB 41663), AGVD2 (NCIMB 41664), AGVD3 (NCIMB 41665) and AGVD17 (NCIMB 41765). *Id.*, pp. 34-35. Applicant describes a breeding process according to the instant invention starting with AGVD1-3 or AGVD17 on page 39 et seq.

Applicant describes crossing the *Sli* gene donor with various potato clones designated: IVP97-079-9, IVPAA-096-I8 and SH83-92-488 to generate clones D1, D2 and D3. *Id.*, p. 49. Applicant describes creating a line containing the *Sli* gene with enhanced agricultural quality in Example 2, pp. 48-51. Applicant describes creating a molecular marker platform, i.e., SNPs, in Example 3, p. 51 et seq. In Example 4, Applicant describes the development of homozygous diploid potato plants and then F1 hybrid progeny, as well as subsequent generations. *Id.*, p. 60 et seq. Applicant describes the meaning of the claim term "essentially homozygous" on pages 21-22 of the instant specification and suggests that the term reflects a level of homozygosity that is above 25% or 75%. *Id.*, p. 21-22. Applicant generally describes adding an agronomically desirable trait to a potato genotype on, e.g. page 37.

The genera also include, insofar as they are listed in claims 20 and 23, the genes providing *P. infestans* resistance such listed in Table 10. *Id.*, pp. 84-91. None of the listed genes are described by deposit – only by reference to an accession number and/or citation to a paper.

Furthermore, Applicant fails to provide, for example, any means to identify the *Sli* gene that is present in the deposited lines. Applicant fails to provide the sequence of the gene, associated genetic markers, the protein encoded by the gene, the chromosomal location of the gene or other equivalent description.

See also *Vas-Cath Inc. v. Mahurkar*, 19 USPQ2d 1111, 1115 (Fed. Cir., 1991), which teaches that the purpose of the written description is for the purpose of warning an innocent purchaser, or other person using a machine, of his infringement of the patent; and at the same time, of taking from the inventor the means of practicing upon the credulity or the fears of other persons, by pretending that his invention is more than what it really is, or different from its ostensible objects, that the patentee is required to distinguish his invention in his specification. In this case, there is no way that a practitioner would be able to determine if the *Sli* gene in a potentially infringing potato line is infringing the instant claims, and therefore, the public has not been put on notice with a sufficient description of the claimed invention.

Applicant fails to describe a representative number of species in comparison to the size of the genera claimed. In the instant application, Applicant describes only four potato lines to use in the claimed methods or as a starting point to obtain the claimed compositions, and all were created from the same line, the IVP07-1001/4 line. *Id.*, pp. 46-48. Applicant fails to describe working examples of the broad genus of other potato lines falling within the scope of the claims. Further, Applicant only describes one parental *S. tuberosum* line and fails to describe examples of claimed potato lines descended from different parental lines.

In the response filed on 14 September 2016, Applicant traverses the rejection under 35 USC 103 that was currently of record and provides background regarding the opinions of artisans in the relevant field. 14 Sept. 2016 Response, pp. 16-22. In support of Applicant's argument against the rejection under 35 USC 103, Applicant filed a declaration under 37 CFR 1.132 by Dr. Lindhout with supporting letters. Dr. Lindhout's declaration was formally filed in application serial no. 13/504,156, and the supporting letters were filed in European Application EP2699684 on 04 June 2014 and are copies therefrom. The letters establish the position that one of skill in the art does not believe Applicant to be in possession of the genus as broadly as claimed even regarding lines similar to Applicant's deposited lines. For example, on page 2 of his letter, Dr. Koornneef states that

> Ever since Lindhout and his co-workers published their results in Potato Research volume 54 of 2011, showing high yielding hybrids from 87% homozygous F3 inbreds, I have been positively surprised that Solynta has managed to challenge this dogma. Solynta could successfully obtain essentially homozygous inbred lines by making use of genotypes that are selfcompatible and by choosing the right starting material that apparently is heterozygous for a lower number of deleterious alleles. This provides the basis of generating useful inbred lines that can become parents of F1 hybrids.

Letter from Koornneef (2012) p. 2 (underlining added). In another letter supplied in support of Dr. Lindhout's declaration, Dr. Jones states that

> "[I]n the mid 2000s we attempted to produce hybrid potato ourselves. . . . However, we never succeeded in developing any promising material in the form of fertile homozygotes that could be useful for hybrid breeding and that could tolerate recurrent selfing. . . . We considered that this was due to our inability to cross the Sli- self-compatibility locus into good enough or diverse enough diploid *Solanum* germplasm.

Letter from Dr. Jonathan D.G. Jones (2012), p. 1 (underlining added). Both letters applaud Applicant for finding potato lines suitable for the instant invention. Conversely, both letters teach that such lines are exceptional.

Applicant claims a large genus of potato plants. Applicant fails to describe a representative number of species in comparison to the size of the genera claimed. Applicant also fails to describe any structure necessary and/or sufficient for a potato line to be used in the claims, i.e. how to obtain *S. tuberosum* lines that are "heterozygous for a lower number of deleterious alleles" (Dr. Koornneef) or "good enough or diverse enough diploid *Solanum* germplasm" (Dr. Jones). Applicant also fails to describe other embodiments of the resistance genes recited in claims 23 and 23 except by improper (*see supra*) reference to the non-patent literature and online databases in Table 10.

The specification therefore fails to provide an adequate written description to support the breadth of the claims. Applicant has not demonstrated possession to one of skill in the art of the instant invention as broadly as claimed at the time of filing, and one of skill in the art would not believe Applicant to be in possession of the genus as broadly as claimed, especially in view of the letters cited above.

Dependent claims are included in this rejection because none provide further limitations obviating this rejection.

Applicant's Arguments

Applicant's position appears to be that the claim amendments obviate the rejection. Response, pp. 23-24.

Applicant points out that the *Sli* gene is present in the deposited lines. *Id.*, p. 23.

Response to Applicant's Arguments

Applicant's statements fail to fully address the rejection. The deposited lines contain the *Sli* gene but Applicant is not claiming *S. tuberosum* lines based solely on the presence of the *Sli* gene. The *Sli* gene, in any case, was known to the art at least by 2005. *See* e.g. Phumichai et al., Genome 48:977-84 (2005).

Claim Rejections - 35 USC § 112(a); enablement

11. Claims 20-21 and 23 are rejected under 35 U.S.C. 112(a) or 35 U.S.C. 112 (pre-AIA), first paragraph, as failing to comply with the enablement requirement. The claim(s) contains subject matter which was not described in the specification in such a way as to enable one skilled in the art to which it pertains, or with which it is most nearly connected, to make and/or use the invention.

The invention appears to employ novel plants or DNA from plants as a source for, e.g. the genes recited in claims 20 and 23. *See* Table 10. Since the plants, or their genes, are essential to the claimed invention they must be obtainable by a repeatable method set forth in the specification or otherwise be readily available to the public. Regarding the required plants, if the plant is not so obtainable or available, the requirements of 35 USC § 112 may be satisfied by a deposit. For example, a deposit of 2,500 seeds of each of the claimed embodiments is considered sufficient to ensure public availability.

Claim 21 is included in this rejection because the Laboratory for Plant Breeding, Wageningen University is not a recognized depository for patents.

The specification does not disclose a repeatable process to obtain the plants and/or DNA and thus it is not apparent if the required plant and/or gene is readily available to the public.

(a) If a deposit is made under the terms of the Budapest Treaty, then a statement, affidavit or declaration by Applicant, or a statement by an attorney of record over his or her signature and registration number, or someone empowered to make such a statement, stating that the instant invention will be irrevocably and without restriction released to the public upon the issuance of a patent, would satisfy the deposit requirement made herein.

(b) If a deposit has not been made under the Budapest Treaty, then in order to certify that the deposit meets the criteria set forth in 37 CFR 1.801-1.809 and MPEP 2402-2411.05, Applicant may provide assurance of compliance by statement, affidavit or declaration, or by someone empowered to make the same, or by a statement by an attorney of record over his or her signature and registration number showing that:

(i) during the pendency of this application, access to the invention will be afforded to the Commissioner upon request;

(ii) all restrictions upon availability to the public will be irrevocably removed upon granting of the patent in accordance with 37 CFR § 1.808(a)(2);

(iii) the deposit will be maintained in a public depository for a period of 30 years or 5 years after the last request or for the effective life of the patent, whichever is longer;

(iv) a test of the viability of the biological material at the time of deposit (see 37 CFR § 1.807); and,

(v) the deposit will be replaced if it should ever become inviable.

In addition, the identifying information set forth in 37 CPR 1.809(d) should be added to the specification, if not already present. See 37 CPR 1.801 - 1.809 (MPEP 2401-2411.05) for additional explanation of these requirements.

Some of the genes listed in Table 10, for example the R5 resistance gene to *P. infestans* from the *S. demissum* on chromosome 11 is only available via a reference, "Huang S (2005)." On page 91 of the specification, Applicant indicates that since this a type "B" entry the gene required for resistance is only characterized by diagnostic markers, presumably in the cited reference. The relevant diagnostic markers are neither described nor taught in the instant specification.

Claim Rejections - 35 USC § 112(a); re: Ex parte Jackson

12. Claims 1-5 and 7-23 are rejected under 35 U.S.C. 112(a) or 35 U.S.C. 112 (pre-AIA), first paragraph, because the specification, while being enabling for the deposited cell lines, does not reasonably provide enablement for forms of self-compatibility as broadly as claimed. The specification does not enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the invention commensurate in scope with these claims.

Due to Applicant's amendments to the claims, the rejection is modified from the rejection set forth in the Office action mailed 22 December 2017 as applied to the

above-referenced claims. Applicant traverses the rejection in the paper filed 23 April 2018. Applicant's arguments have been fully considered but are not persuasive.

The invention appears to employ plants with novel characteristics. Since these plant characteristics are essential to the claimed invention it must be obtainable by a repeatable method set forth in the specification or otherwise be readily available to the public. If the plant is not so obtainable or available, the requirements of 35 USC § 112 may be satisfied by a deposit of the seeds. Deposits under NCIMB accession numbers 41663, 41664, 41665 and 41765 have been made. However the claims are not drawn solely to the deposits.

Claim 1 is drawn to a diploid, fertile, self-compatible and essentially homozygous *Solanum tuberosum* potato line having an average tuber yield expressed in grams of fresh weight of at least 200 grams per plant; wherein said line additionally comprises 1-6 agronomically desirable traits or genes where the trait provides resistance to *P. infestans* and wherein the self-compatibility originates from *S. chacoense* and is present in a plant of potato lines NCIMB accession number 41663, NCIMB accession number 41664, NCIMB accession number 41665, or NCIMB accession number 41765, further where representative samples were deposited with the NCIMB under breeders reference AGVD1, AGVD2, AGVD3, and AGVD17.

Claims 2-4 depending from claim 1 read on a hybrid potato plant or seed produced from a cross between two essentially homozygous parental breeding lines and a potato plant(s) produced therefrom. Claim 8 reads on a method using a plant from claim 1. Claims 9-13 read on plants and plant parts produced by the method of claim 8. Claim 14 reads on a tissue culture produced by the plant of claim 10 and claim

15 reads on a plant produced from the tissue culture of claim 14. Claim 16 reads on a method using a seed produced according to claim 8 to produce a plant; claim 17 reads on a plant or tuber produced according to claim 16. Claim 18 reads on a set of at least 2 intercrossable diploid, fertile, self-compatible and essentially homozygous *S. tuberosum* potato lines according to claim 1.

New claims 20-22 depend from claim 1. Dependent claim 20 recites various embodiments of genes encoding *P. infestans* resistance. Dependent claim 21 recites that the potato line used is a progeny of a line available at a laboratory at Wageningen University. Dependent claim 22 recites that the potato line used is a progeny of a deposited line.

Claim 5 is a method claim drawn to producing a diploid, fertile, self-compatible and essentially homozygous *Solanum tuberosum* potato line starting with a first potato plant of a first diploid self-compatible, fertile and essentially homozygous potato line wherein the self-compatibility originates from *S. chacoense* and is present in a plant of potato lines NCIMB accession number 41663, NCIMB accession number 41664, NCIMB accession number 41665, or NCIMB accession number 41765, further where representative samples were deposited with the NCIMB under breeders reference AGVD1, AGVD2, AGVD3, and AGVD17 and crossing it to a second potato plant where the second potato plant has an average tuber yield expressed in grams of fresh weight of at least 200 grams per plant and then selfing or backcrossing to provide a diploid, self-compatible and essentially homozygous *S. tuberosum* potato breeding line comprising plants having an average tuber yield of at least 200 grams per plant, and

then introducing into a plant of said line at least one trait or gene where the gene or trait provides resistance to *P. infestans*.

Dependent claim 6 recites specific deposited cell lines and is not rejected under the written description requirement; claim 7 encompasses a diploid potato breeding line obtained by the method of claim 5. Claim 19 reads on an elite diploid potato breeding line obtained by the method of claim 5. New claim 23 depends from claim 5 and recites various embodiments of genes encoding *P. infestans* resistance.

The claimed genera, in particular, include those set forth in the following exemplary claims:

claim 1 – any "diploid, fertile, self-compatible and essentially homozygous Solanum tuberosum potato line" wherein it has a tuber yield of at least 200 g per plant and wherein the self-compatibility gene originates from S. chacoense and is present in a plant of potato lines NCIMB accession number 41663, NCIMB accession number 41664, NCIMB accession number 41665, or NCIMB accession number 41765;

claim 5 – any "diploid, fertile, self-compatible and essentially homozygous Solanum tuberosum potato line" wherein it has a tuber yield of at least 200 g per plant and wherein the self-compatibility gene originates from S. chacoense and is present in a plant of potato lines NCIMB accession number 41663, NCIMB accession number 41664, NCIMB accession number 41665, or NCIMB accession number 41765 as a starting material in part (a) to produce a "diploid, fertile, self-compatible and essentially homozygous Solanum tuberosum potato line" with an added trait or gene, wherein claim 5 is a method claim but several dependent claims read on compositions produced by the method.

The above claims, however, are not limited to potato lines described by the deposits. The claims read on all "diploid, fertile, self-compatible and essentially homozygous *Solanum tuberosum* potato" lines with a tuber yield of 200 g – but only the deposited lines are taught.

The Federal Circuit in *In re Wands* lists eight considerations for determining whether or not undue experimentation would be necessary to practice an invention. *In re Wands*, 858 F2d 731, 8 USPQ2d 1400, 1406 (Fed. Cir. 1988). These factors are: the quantity of experimentation necessary, the amount of direction or guidance presented, the presence or absence of working examples of the invention, the nature of the invention, the state of the prior art, the relative skill of those in the art, the predictability or unpredictability of the art, and the breadth of the claims. *Id.*

In the specification, Applicant teaches obtaining the IVP07-1001/4 line from publically available germplasm and plants that contain the *Sli* gene and using these plants to breed AGVD1, AGVD2, and AGVD3. Spec., pp. 46-47. Applicant teaches that lines were deposited and that the deposited lines are known by their breeders reference: AGVD1 (NCIMB 41663), AGVD2 (NCIMB 41664), AGVD3 (NCIMB 41665) and AGVD17 (NCIMB 41765). *Id.*, pp. 34-35. Applicant teaches a breeding process according to the instant invention starting with AGVD1-3 or AGVD17 on page 39 et seq.

Applicant teaches crossing the *Sli* gene donor with various potato clones designated IVP97-079-9, IVPAA-096-I8 and SH83-92-488 to generate clones D1, D2 and D3. *Id.*, p. 49. Applicant teaches creating a line containing the *Sli* gene with enhanced agricultural quality in Example 2, pp. 48-51. Applicant teaches creating a molecular marker platform, i.e., SNPs, in Example 3, p. 51 et seq. In Example 4,

Applicant teaches the development of homozygous diploid potato plants and then F1 hybrid progeny, as well as subsequent generations. *Id.*, p. 60 et seq. Applicant teaches the meaning of the claim term "essentially homozygous" on pages 21-22 of the instant specification and suggests that the term reflects a level of homozygosity that is above 25% or 75%. *Id.*, p. 21-22. Applicant generally teaches adding an agronomically desirable trait to a potato genotype on, e.g. page 37.

The art generally teaches this breeding strategy, or similar strategies. For example, Jansky teaches first obtaining homozygous diploids using a self-incompatibility inhibitor. J Jansky, "Breeding, Genetics, and Cultivar Development," Advances in Potato Chemistry and Technology, ed. J. Singh & L. Kaur, pp. 27-62 (June, 2009), p. 46. Jansky teaches that "[h]ighly homozygous inbreds could be also be used for heterosis breeding. Jansky, p. 46. Jansky teaches that such an approach would be difficult. *Id.*

Phumichai et al. confirms the approach of Jansky, and documents the use of the *Sli* gene in the approach. Phumichai et al., Genome 48:977-84 (2005), abstract.

> Potato (Solanum tuberosum) is an auto- [sic] or segmental allo-tetraploid showing tetrasomic inheritance, which results in complicated genetic segregation. Since potatoes are propagated vegetatively, highly heterotic genotypes can be maintained, which facilitates the use of heterosis, an essential factor in potato breeding, particularly for tuber yield. Alternatively, open-pollinated true potato seed (TPS) can be used as a low-cost planting material for potato production.
>
> Highly homozygous inbreds, once amply produced, would be highly useful for the development of TPS varieties with genetic uniformity, as well as for the investigation of heterosis."
>
> "A scheme for hybrid breeding using homozygous diploids and subsequent somatic hybridization has been proposed, for which the availability of highly homozygous diploid (or pure) lines is a key for success. In considering the use of pure lines for the development of

> genetically uniform [true potato seed] varieties, self-fertile, seed-propagated lines are highly desirable, and simple techniques to develop such lines are necessary." .

Id., pp. 977-78 (citations omitted).

In the response filed on 14 September 2016, Applicant traverses the rejection of record at that time under 35 USC 103 and argues that one of skill in the art would not have a reasonable expectation of success in obtaining lines such as those deposited. Response, pp. 16-22. As part of this argument, Applicant reviews the scope and content of the prior art, including the references cited above. For example, Applicant cites to a publication from Watanabe et al. (Breed Sci 46:329-36 (1996)) as standing for "homozygous inbred lines have not been obtained at the diploid level due to both inbreeding depression and self-incompatibility." Response, p. 17. Applicant also cites to Jansky as downplaying the possibility of hybrid breeding as 'commercially unfeasible due to "dramatic reductions in fertility and vigor.'" Response, p. 17 (quoting Jansky, p. 46). Applicant also argues that Uijtewaal et al. (Euphytica, 36: 745-753, 1987) confirms this teaching. Response, p. 17 & p. 19. Applicant concludes the argument with citation of additional material supporting their position that one of skill in the art would not have a reasonable expectation of success in combining the art cited in the rejection under 35 USC 103.

In support of the argument, Applicant also filed a declaration under 37 CFR 1.132 by Dr. Lindhout with supporting letters. Dr. Lindhout's declaration was formally filed in application serial no. 13/504,156, and the supporting letters were initially filed in European Application EP2699684 (04 June 2014) and are copies therefrom. The letters

teach that the success of Applicant's invention, as broadly as claimed, was unpredictable. For example, on page 2 of his letter, Dr. Koornneef states that

> Ever since Lindhout and his co-workers published their results in Potato Research volume 54 of 2011, showing high yielding hybrids from 87% homozygous F3 inbreds, I have been positively surprised that Solynta has managed to challenge this dogma. Solynta could successfully obtain essentially homozygous inbred lines by making use of genotypes that are selfcompatible and by choosing the right starting material that apparently is heterozygous for a lower number of deleterious alleles. This provides the basis of generating useful inbred lines that can become parents of F1 hybrids.

Letter from Koornneef (2012) p. 2 (underlining added). Earlier, Dr. Koornneef also states that "to start a project aiming at developing a good performing homozygous potato plant for the purpose of hybrid production is a daring undertaking with very little chance of success. *Id.*

In another letter supplied in support of Dr. Lindhout's declaration, Dr. Jones states that

> "[I]n the mid 2000s we attempted to produce hybrid potato ourselves. . . . However, we never succeeded in developing any promising material in the form of fertile homozygotes that could be useful for hybrid breeding and that could tolerate recurrent selfing. . . . We considered that this was due to our inability to cross the Sli- self-compatibility locus into good enough or diverse enough diploid *Solanum* germplasm.

Letter from Dr. Jonathan D.G. Jones (2012), p. 1 (underlining added).

Applicant claims a large genus of potato plants. Jansky and Spooner et al. describes the breadth of the of the claimed genus as extremely large. See, e.g., Jansky, "Breeding, Genetics, and Cultivar Development," Advances in Potato Chemistry and Technology, ed. J. Singh & L. Kaur, pp. 27-62 (June, 2009), pp. 27-29; Spooner et al., Bot Rev 80:283-383 (2014), Table 1, pp. 286-94. Applicant's claimed invention reads on any potential potato line that is a "diploid, fertile, self-compatible and

essentially homozygous *Solanum tuberosum* potato line" with a tuber yield of 200 g where the *Sli* gene is in the deposited lines.

In view of Applicant's arguments in response to the rejection under 35 USC 103 and Dr. Lindhout's supporting letters, success in practicing Applicant's claimed invention would be unpredictable and would depend on the genotype of the potato lines. Therefore, extensive guidance would be required to enable the invention as broadly as claimed. Such guidance is not provided.

The specification therefore does not enable one skilled in the art to make and use members of the species of the claimed genus other than the deposited lines. See, for example, MPEP 2164.06(a)(II), citing to *Ex parte Jackson*, 217 USPQ 804, 806 (Bd. App. 1982). The issue before the Board in *Ex parte Jackson* was:

> The issue squarely raised by this rejection is whether or not a description of several newly discovered strains of bacteria having one particularly desirable metabolic property in terms of the conventionally measured culture characteristics and a number of metabolic and physiological properties would enable one of ordinary skill in the relevant art to independently discover additional strains having the same specific, desirable metabolic property, i.e., the production of a particular antibiotic.
>
> To state the issue somewhat differently, it is whether a verbal description of a new species would enable one of ordinary skill in the relevant art to obtain strains of that species over and above the specific strains made available through deposit in one of the recognized culture depositories.

Ex parte Jackson, 217 USPQ 804, 806 (Bd. App. 1982). In the instant application, the issue is similar to the one before the Board in that Applicant made deposits with unique characteristics but not taught how to identify other lines. In *Ex parte Jackson*, the Board held that the deposit, without a detailed explanation of how to obtain more species from nature, did not enable the generic claims. *Id.*, p. 808. The board found that "the degree of experimentation involved in locating new [organisms] apart from deposited cultures is

undue in light of the enablement requirement of 35 U.S.C. 112." *Id.* The *Jackson* decision was considered and distinguished by the Federal Circuit in the *Wands* decision in view of the routine experimentation required to obtain hybridomas. *In re Wands*, pp. 1403-04.

The claimed but not enabled genera also include, insofar as they are listed in claims 20 and 23, the genes listed in Table 10. On page 7 of the amended specification submitted 23 April 2017, Applicant indicates that some of the genes listed in Table 10, for example resistance to *P. infestans* from the *R6* gene (claim 20, l. 8) is only provided by reference to a non-patent literature document. The entries in Table 10, insofar as the provide matter essential to determining the scope of the claims, are not enabled.

Applicant fails to enable the invention as broadly claimed. For example, the Supreme Court held that "[h]he basic quid pro quo contemplated by the Constitution and the Congress for granting a patent monopoly is the benefit derived by the public from an invention with substantial utility. Unless and until a process is refined and developed to this point--where specific benefit exists in currently available form--there is insufficient justification for permitting an applicant to engross what may prove to be a broad field." *Brenner v. Manson*, 383 U.S. 519, 148 USPQ 689, 695 (1966). Applicant teaches only four embodiments. In view of, for example, the surprise of Dr. Koornneef and Dr. Jones, Applicant fails to provide the public with the knowledge of how to practice Applicant's invention with the exception of the embodiments of the deposited lines.

Therefore, in view of the *Ex parte Jackson* decision, the generic scope of, e.g. claims 1 and 5, reading on the genera discussed above, is not enabled. Obtaining

further species falling within the scope of the generic claim would require undue experimentation.

Dependent claims are included in this rejection because none provide further limitations obviating this rejection.

Applicant's Arguments

Applicant's position appears to be that the claim amendments obviate the rejection and that the claims are solely drawn to the deposit. Response, p. 25. Applicant points out that the *Sli* gene is present in the deposited lines. *Id.*

Response to Applicant's Arguments

Applicant's statements fail to fully address the rejection. The deposited lines contain the *Sli* gene but Applicant is not claiming *S. tuberosum* lines based solely on the presence of the Sli gene. The *Sli* gene, in any case, was known to the art at least by 2005. See e.g. Phumichai et al., Genome 48:977-84 (2005).

Furthermore, the examiner disagrees with Applicant's assertion that "no degree of experimentation is involved in locating the genes from the deposited lines." Significant experimentation would be required.

Conclusion

No claim is allowed.

Applicant's amendment necessitated the new ground(s) of rejection presented in this Office action. Accordingly, **THIS ACTION IS MADE FINAL**. See MPEP

§ 706.07(a). Applicant is reminded of the extension of time policy as set forth in 37 CFR 1.136(a).

A shortened statutory period for reply to this final action is set to expire THREE MONTHS from the mailing date of this action. In the event a first reply is filed within TWO MONTHS of the mailing date of this final action and the advisory action is not mailed until after the end of the THREE-MONTH shortened statutory period, then the shortened statutory period will expire on the date the advisory action is mailed, and any extension fee pursuant to 37 CFR 1.136(a) will be calculated from the mailing date of the advisory action. In no event, however, will the statutory period for reply expire later than SIX MONTHS from the date of this final action.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to RUSSELL T BOGGS whose telephone number is (571)272-2805. The examiner can normally be reached on Monday - Friday, 0800 to 1830 Mtn.

Examiner interviews are available via telephone, in-person, and video conferencing using a USPTO supplied web-based collaboration tool. To schedule an interview, applicant is encouraged to use the USPTO Automated Interview Request (AIR) at http://www.uspto.gov/interviewpractice.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Amjad Abraham can be reached on 571-270-0708. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free). If you would like assistance from a USPTO Customer Service Representative or access to the automated information system, call 800-786-9199 (IN USA OR CANADA) or 571-272-1000.

/RUSSELL T BOGGS/
Examiner, Art Unit 1663

I hereby certify that this paper (along with any paper referred to as being attached or enclosed) is being transmitted via the Office electronic filing system in accordance with 37 CFR § 1.6(a)(4).

Dated: _2/7/2019_____ Signature: __/Kristin A. Connarn/____

Docket No.: 091876-0027 **PATENT**

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of: Willem Hendrik LINDHOUT, et al.	:	Customer Number: 23630
Application No.: 14/112,747	:	Confirmation No.: 5919
Filed: December 23, 2013	:	Art Unit: 1663
For: Hybrid Seed Potato Breeding	:	Examiner: Russell T. BOGGS

REQUEST FOR CONTINUED EXAMINATION AND AMENDMENT IN RESPONSE TO FINAL OFFICE ACTION

Mail Stop - Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

INTRODUCTORY COMMENTS

In response to the non-final Office Action dated August 7, 2018, please amend the above-identified U.S. patent application as directed herein. In addition, this Amendment is accompanied by a Request for Continued Examination under 37 C.F.R. § 1.114 and Petition for three-month extension of time. Reconsideration and further examination of the present application is requested in light of the following.

Amendments to the Claims begin on Page 2.

Remarks/Arguments begin on Page 7.

AMENDMENTS TO THE CLAIMS

1. (Currently Amended) A diploid, fertile, self-compatible and essentially homozygous *Solanum tuberosum* potato line comprising plants having an average tuber yield expressed in grams of fresh weight of at least 200 grams per plant, ~~wherein said line in addition to said tuber yield comprises at least one, more preferably at least 2, 3, 4, 5 or 6 agronomically desirable traits or genes,~~ wherein said self-compatibility originates from *Solanum chacoense* and is referred to as S-locus inhibitor (*Sli*) gene, ~~a copy of said gene~~ as is present in a plant of potato lines NCIMB accession number 41663, NCIMB accession number 41664, NCIMB accession number 41665, or NCIMB accession number 41765, representative seeds of said lines having been deposited with the NCIMB, Aberdeen, Scotland under breeders reference AGVD1, AGVD2, AGVD3, and AGVD17 respectively, wherein said line in addition to said tuber yield comprises at least one agronomically desirable trait or gene, ~~and~~ wherein said at least one agronomically desirable trait or gene provides resistance to *Phytophthora infestans* infections.

2. (Previously Presented) A hybrid potato seed resulting from a cross between two essentially homozygous parental potato breeding lines, at least one of which is a plant of the line according to claim 1.

3. (Previously Presented) A hybrid potato plant grown from the seed of claim 2.

4. (Previously Presented) A tetraploid plant produced by doubling the genome of a plant of the line of claim 1.

5. (Currently Amended) A method for producing a diploid, fertile, self-compatible and essentially homozygous *Solanum tuberosum* potato line comprising plants having an average tuber yield expressed in grams of fresh weight of at least 200 grams per plant, comprising:

(a) providing a first potato plant, which first potato plant is a plant of a first diploid, self-compatible~~,~~ and fertile ~~and essentially homozygous~~ potato line, wherein said self-compatibility

originates from *Solanum chacoense* and is referred to as S-locus inhibitor (*Sli*) gene, ~~a copy of said gene~~ as is present in a plant of potato lines NCIMB accession number 41663, NCIMB accession number 41664, NCIMB accession number 41665, or NCIMB accession number 41765, representative seeds of said lines having been deposited with the NCIMB, Aberdeen, Scotland under breeders reference AGVD1, AGVD2, AGVD3, and AGVD17 respectively;

(b) providing a second potato plant, wherein said second potato plant is a plant of a diploid or tetraploid *Solanum tuberosum* potato line which may have any level of homozygosity, said line of said second potato plant comprising plants having an average tuber yield expressed in grams of fresh weight of at least 200 grams per plant;

(c) cross pollinating said first and second potato plant to provide seeds and collecting said seeds to thereby provide an offspring generation in the form of a hybrid offspring seed;

(d) growing said hybrid offspring seed into a population of hybrid offspring potato plant and selecting from said population plants being fertile and producing tubers at a yield expressed in grams of fresh weight of at least 200 grams per plant, and

(e) selfing said hybrid offspring potato plant or backcrossing said hybrid offspring potato plant to a potato plant of said line of said first potato plant, for between 1 to 8 selfings or backcrosses, to thereby provide a diploid, self-compatible and essentially homozygous *Solanum tuberosum* potato breeding line comprising plants having an average tuber yield expressed in grams of fresh weight of at least 200 grams per plant;

(f) said method further comprising the step of introducing into a plant of said diploid, self-compatible and essentially homozygous potato breeding line obtained in step (e) at least one trait or gene provides resistance to *Phytophthora infestans* infections.

6. (Previously presented) The method of claim 5, wherein said first potato plant is a plant of potato line NCIMB accession number 41663, NCIMB accession number 41664, NCIMB accession number 41665, or NCIMB accession number 41765, representative seeds of said line having been deposited with the NCIMB, Aberdeen, Scotland under breeders reference AGVD1, AGVD2, AGVD3, and AGVD17 respectively.

7. (Previously presented) A diploid potato breeding line obtained by the method of claim 5.

8. (Previously Presented) A method for producing a uniform hybrid potato seed, comprising:

(a) providing a first potato plant of the line according to claim 1,

(b) providing a second potato plant, wherein said second potato plant is a plant of a diploid, fertile, self-compatible and essentially homozygous potato line, and

(c) cross pollinating said first and second potato plant and allowing one of either said first or second potato plant to produce berries with seeds and collecting said seeds from said berries to provide an offspring generation in the form of a hybrid offspring seed.

9. (Original) A seed produced by the method of claim 8.

10. (Original) A hybrid potato plant, or part thereof, produced by growing the seed of claim 9.

11. (Previously Presented) A pollen or an ovule of the plant of claim 10.

12. (Original) A tuber from the plant of claim 10.

13-17. (Cancelled)

18. (Previously Presented) A set of at least 2 intercrossable diploid, fertile, self-compatible and essentially homozygous potato lines according to claim 1.

19. (Previously presented) An elite diploid potato breeding line obtained by the method of claim 5.

20. (Previously Presented) The diploid, fertile, self-compatible and essentially homozygous *Solanum tuberosum* potato line according to claim 1, wherein said at least one additional trait or gene that provides resistance to *Phytophthora infestans* infections is selected from the group

consisting of *Solanum demissum* gene *R1* on chromosome 5; *S. demissum* gene *R2* on chromosome 4; *Solanum sp.* AM-3778-16 gene *R2*-like on chromosome 4; *S. bulbocastanum* gene *Rpi-blb1/Rb* on chromosome 8; *S. bulbocastanum* gene *Rpi-blb2* on chromosome 6; *S. bulbocastanum* gene *Rpi-bt1* on chromosome 8; *S. demissum* gene *R5* on chromosome 11; *S. demissum* gene *R6* on chromosome 11; *S. demissum* gene *R7* on chromosome 11; *S. demissum* gene *R9* on chromosome 11; *S. demissum* gene *R10* on chromosome 11; *S. demissum* gene *R11* on chromosome 11; *S. hjertingii* genes *Rpi-hjt1.1*, *Rpi-hjt1.2* and *Rpi-hjt1.3* on chromosome 4; *S. bulbocastanum Rpi-abpt* gene on chromosome 4; *S. venturii* genes *Rpi-vnt1.1*, *Rpi-vnt1.2*, and *Rpi-vnt1.3* on chromosome 9; *S. demissum* gene *R3a* on chromosome 11; *S. demissum* potato gene *R3b* on chromosome 11; *S. pinnatisectum* gene *Rpi1* on chromosome 7; *S. berthaultii* gene *Rpi-ber1* on chromosome 10; and *S. microdontum* gene *Rpi-mcd1* on chromosome 4.

21. (Cancelled)

22. (Previously Presented) The diploid, fertile, self-compatible and essentially homozygous *Solanum tuberosum* potato line according to claim 1, wherein said potato line is a progeny line of potato line NCIMB 41663, NCIMB 41664, NCIMB 41665, or NCIMB 41765, representative seeds of said NCIMB lines having been deposited with the NCIMB, Aberdeen, Scotland under breeders reference AGVD1, AGVD2, AGVD3, and AGVD17 respectively.

23. (Previously Presented) The method according to claim 5, wherein the at least one trait or gene that is introduced into the plant of said diploid, self-compatible and essentially homozygous potato breeding line obtained in step (e) is selected from the group consisting of *Solanum demissum* gene *R1* on chromosome 5; *S. demissum* gene *R2* on chromosome 4; *Solanum sp.* AM-3778-16 gene *R2*-like on chromosome 4; *S. bulbocastanum* gene *Rpi-blb1/Rb* on chromosome 8; *S. bulbocastanum* gene *Rpi-blb2* on chromosome 6; *S. bulbocastanum* gene *Rpi-bt1* on chromosome 8; *S. demissum* gene *R5* on chromosome 11; *S. demissum* gene *R6* on chromosome 11; *S. demissum* gene *R7* on chromosome 11; *S. demissum* gene *R9* on chromosome 11; *S. demissum* gene *R10* on chromosome 11; *S. demissum* gene *R11* on chromosome 11; *S. hjertingii* genes *Rpi-hjt1.1*, *Rpi-*

hjt1.2 and *Rpi-hjt1.3* on chromosome 4; *S. bulbocastanum* Rpi-abpt gene on chromosome 4; *S. venturii* genes *Rpi-vnt1.1*, *Rpi-vnt1.2*, and *Rpi-vnt1.3* on chromosome 9; *S. demissum* gene *R3a* on chromosome 11; *S. demissum* potato gene *R3b* on chromosome 11; *S. pinnatisectum* gene *Rpi1* on chromosome 7; *S. berthaultii* gene *Rpi-ber1* on chromosome 10; and *S. microdontum* gene *Rpi-mcd1* on chromosome 4.

24. (New) The method according to claim 5, wherein the at least one trait or gene that is introduced into the plant of said diploid, self-compatible and essentially homozygous potato breeding line obtained in step (e) is selected from *S. venturii* genes *Rpi-vnt1.1*, *Rpi-vnt1.2,* and *Rpi-vnt1.3* on chromosome 9.

25. (New) The method according to claim 5, wherein the at least one trait or gene that is introduced into the plant of said diploid, self-compatible and essentially homozygous potato breeding line obtained in step (e) is introduced by transgenic means.

26. (New) The diploid, fertile, self-compatible and essentially homozygous *Solanum tuberosum* potato line of claim 1, wherein at least 25% of the homozygous genomic loci in a plant of said line are from one or more of NCIMB accession number 41663, NCIMB accession number 41664, NCIMB accession number 41665, and NCIMB accession number 41765.

REMARKS/ARGUMENTS

Status of Claims

Claims 1 and 5 have been amended. Claims 13-17 and 21 have been cancelled. New claims 24-26 have been added.

Claim 1 has been amended to refer to the introduction of at least one agronomically desirable trait or gene that provides resistance to *Phytophthora infestans* infections. Claim 1 is further amended to refer to the "(*Sli*) gene, as is present in a plant."

Claim 5 has been amended to delete the phrase "essentially homozygouos" in step (a) and to indicate in step (d) that selection includes selection of fertile plants. Claim 5 is further amended to refer to the "(*Sli*) gene, as is present in a plant."

New claim 24 has been added to refer to preferred *Phytophthora* resistance genes Rpi-vnt1.1, Rpi-vnt1.2, and Pri-vnt1.3 on chromosome 9 of *S. venturii.*

New claim 25 has been added, indicating that the at least one trait or gene can be introduced by transgenic means. Support for this claim is found, for example, on page 35, lines 24-27.

New claim 26 has been added, indicating that the at least 25% of the homozygous genomic loci in a plant of the line according to claim 1 are from one or more of NCIMB accession number 41663, NCIMB accession number 41664, NCIMB accession number 41665, and NCIMB accession number 41765.

Objection to the Specification

The Examiner has objected to the specification because in his opinion it is the Applicant's clear intent to incorporate the essential material cited by reference to the non-patent literature listed in Table 10 by reference to the non-patent literature into the claims. The Examiner states that incorporation of essential material into the specification by reference to an unpublished U.S. application, foreign application or patent, to a publication, or to an online database is improper. The

Examiner suggests amendment of claims 20 and 23 to remove all reference to material only defined by reference to Table 10 would obviate this objection, because then it would no longer be essential material.

Applicant respectfully disagrees. The traits or genes that provide resistance to *Phytophthora infestans* infections and which are included in dependent claims 20 and 23 were present in original Table 10 of the underlying PCT application, published as WO2012/144902. It is not Applicant's intent, nor is it necessary, to incorporate the non-patent literature cited in Table 10.

Rejections under 35 U.S.C. § 112 (b), second paragraph

Claims 1-23 are rejected under 35 U.S.C. § 112 (b), second paragraph, as being indefinite, for failing to particularly point out and distinctly claim the subject matter which Applicant regards as the invention.

Claim 1 is rejected because it recites the limitation "said gene" in line 2 and the antecedent basis for this limitation is ambiguous. Claim 1 is further rejected because it recites the limitation "said at least one agronomically desirable trait or gene" in line 10 and the antecedent basis for this limitation is ambiguous. Claim 1 has been amended to remove the ambiguity.

Claim 21 is rejected because it is drawn to a progeny line of a line available at a specific laboratory at Wageningen University. Claim 21 has been cancelled, thereby making this rejection moot.

Claims 20 and 23 are rejected because they recite numerous limitations in which genes, for example, are identified by a variety of terms where the meaning of the terms are indefinite. In the Examiner's opinion, these terms lack general art-accepted meanings and Applicant does not explicitly define the terms in the specification, for example, by reference to the sequence listing or by reference to a specific location in a deposited cell line. Applicant respectfully disagrees with the Examiner that the terms lack general art-accepted meanings. The traits and genes listed in claims 20 and 23 were known to a person skilled in the art. For example, the *Solanum demissum* genes have been known in the art for more than 60 years (see Black et al., 1953. Euphytica 2:173-178; Malcolmson and Black, 1966. Euphytica 15:199-203). Table 10 provides reference to scientific

publications that describe the identification of these genes. The genes have subsequently been used in crosses that were reported in numerous scientific publications. Hence, a skilled person is, without doubts, able to introduce one or more of the listed *Phytophthora infestans* resistance traits or genes into a plant of a diploid, fertile, self-compatible *Solanum tuberosum* potato line. Applicant stresses that in contrast to Examiner's conclusion here and in objecting to the specification, the reference to the specific traits and genes is not material, as the claims are not directed to the *Phytophthora* resistance genes themselves, but rather to the potato lines having the specific traits or genes recited in claims 20 and 23.

Reconsideration and withdrawal of rejection are respectfully requested.

Rejections under 35 U.S.C. § 112(a), written description

Claims 1-5 and 7-23 are rejected under 35 U.S.C. 112(a) or 35 U.S.C. 112 (pre-AIA), first paragraph, as failing to comply with the written description requirement.

According to the Examiner, the claims are drawn to a diploid, fertile, self-compatible and essentially homozygous *Solanum tuberosum* potato line having an average tuber yield expressed in grams of fresh weight of at least 200 grams per plant; wherein said line additionally comprises 1-6 agronomically desirable traits or genes where the trait provides resistance to *P. infestans* and wherein the self-compatibility originates from *S. chacoense* and is present in a plant of potato lines NCIMB accession number 41663, NCIMB accession number 41664, NCIMB accession number 41665, or NCIMB accession number 41765, further where representative samples were deposited with the NCIMB under breeders reference AGVD1, AGVD2, AGVD3, and AGVD17. In the Examiner's opinion, the specification fails to provide an adequate written description to support the breadth of the claims. The Examiner concludes that the Applicant has not demonstrated possession to one skilled in the art, and one of skill in the art would not believe Applicant to be in possession of the genus as broadly as claimed, in view of the letters previously provided.

Applicant respectfully disagrees. The Examiner indicates that the application as filed does not provide a representative number of species to claim a broad genus of self-compatible *Solanum tuberosum* potato lines. As described in the Declaration of Pim Lindhout, filed in a supplementary

response to the co-pending application US 13/504,156, the inventors were in possession of the claimed genus at the time of filing. Whole genome sequencing data shows that the genomic variation between lines D1, D2, and D3 is extremely large and represents virtually all variation available in *S. tuberosum*. In fact, the genomic variation is wider than that within tetraploid *S. tuberosum*. Hence, by showing that the three diploid *S. tuberosum* lines D1-D3 can all successfully be used as starting material, we have shown that there are no limits as to the starting material. Indpendent research, using completely different diploid *S. tuberosum* clones as recipients of the *Sli*-gene, has confirmed that it is possible to generate fertile, essentially homozygous *S. tuberosum* potato lines when selecting for fertile offspring in selfed progenies (Jansky et al., 2014 J. Plant Registrations. Doi:10.3198/jpr2013.05.0024.crg; Jansky et al., 2016. Crop Sci 56:1412-1422).

Thus, the specific starting material of D1-D3 is representative of the genus claimed. The specific lines D1, D2, and D3 are exemplary, but not essential to the success of the claimed method of producing the potato plants.

Reconsideration and withdrawal of rejection are respectfully requested.

Rejections under 35 U.S.C. § 112(a), enablement

Claims 20, 21, and 23 are rejected under 35 U.S.C. 112(a) or 35 U.S.C. 112 (pre-AIA), first paragraph, as failing to comply with the enablement requirement. In the Examiner's opinion the invention appears to employ novel plants or DNA from plants as a source for the genes recited in claims 20 and 23 (See Table 10), thus the plants or their genes are considered essential to the claimed invention. Such essential material must be obtainable by a repeatable method set forth in the specification or otherwise be readily available to the public. If the plant is not readily obtainable or available, the requirements of 35 USC § 112 may be satisfied by deposit. The Examiner further states that some of the genes listed in Table 10 are available only via a reference (e.g., Huang S (2005)).

Applicant respectfully submits that the novel plants and DNA from plants as a source for the genes recited in claims 20 and 23 were deposited under the terms of the Budapest Treaty. A

statement was provided in the response of September 14, 2016 stating that the instant invention will be irrevocably and without restriction released to the public upon the issuance of a patent. Thus, Applicant believes the deposit requirement has already been met with respect to claims 20 and 23.

Claim 21 has been cancelled, thereby making this rejection moot.

The claims refer to a diploid, fertile, self-compatible and essentially homozygous *Solanum tuberosum* potato line. Said self-compatibility originates from *S. chacoense* and is referred to as *S-locus inhibitor (Sli) gene*. This gene is present in a plant of potato lines under NCIMB accession numbers 41663, 41664, 41665, and 41765. The claims do not recite the *Sli* gene itself. The claims recite self-compatible *Solanum tuberosum* potato lines. After the first description of the *Sli* gene by Hanneman in 1985, the gene was mapped at a distal end of chromosome 12 by Hosaka and Hanneman in 1998 (Hosake and Hanneman, 1998. Euphytica 103:265-271). Since then, numerous groups have successfully introgressed this gene in *Solanum tuberosum* potato lines to render these lines self-comptabile.

The presence of the *Sli*-gene can easily be determined by a skilled person by testing for self-compatiblity. If needed, one may use markers, but such markers were not used by the present inventors, nor were they used by other researchers who availed themselves of the *Sli*-gene in order to convert self-incompatible potato plants into self-compatible plants (e.g., Hosake and Hanneman, 1998. Euphytica 99:191-197; Birhman and Hosaka, 2000, Genome 43: 495-502; Phymichai et al., 2005, Genome 48: 977-984). A practitioner is thus able to determine the presence or absence of the *Sli* gene in a self-compatible potato line.

As for the *Phytophthora* resistance genes, as is indicated herein above, the traits or genes that provide resistance to *Phytophthora infestans* infections and which are included in dependent claims 20 and 23, were known to a person skilled in the art. Said genes have been described in the art previously, and are also provided for example in the cited references provided in Table 10. Thus, a skilled person is, without doubt, able to introduce one or more of the listed *Phytophthora infestans* resistance traits or genes into a plant of a diploid, fertile, self-compatible and essentially homozygous *Solanum tuberosum* potato line.

Reconsideration and withdrawal of rejection are respectfully requested.

Rejections under 35 U.S.C. § 112(a), re: Ex parte Jackson

Claims 1-5 and 7-23 are rejected under 35 U.S.C. 112(a) or 35 U.S.C. 112 (pre-AIA), first paragraph, because the specification while enabling for the deposited lines, does not reasonably provide enablement for forms of self-compatibility. In the Examiner's opinion, the specification would not enable one skilled in the art to make and use members of the species of the claimed genus other than the deposited lines.

Applicant respectfully disagree. The application as filed teaches one of skill in the art to produce lines falling under the scope of the claim and provides working examples of *Solanum tuberosum* plants that are diploid, fertile, self-compatible and essentially homozygous. A key to Applicant's success, and something clearly taught in the specification, was the selection of fertile offspring in every (backcross) generation, rather than selection based on tuber yield, as was routine in potato breeding. As is indicated herein above, independent research has confirmed that it is possible to generate fertile, essentially homozygous *S. tuberosum* potato lines when selecting for fertile offspring in selfed progenies (Jansky et al., 2014 J. Plant Registrations. doi: 10.3198/jpr2013.05.0024crg; Jansky et al., 2016. Crop Sci 56:1412-1422).

Reconsideration and withdrawal of rejection are respectfully requested.

CONCLUSION

Should the Examiner be of the view that an interview would expedite consideration of the application, request is made that the Examiner telephone the Applicant's attorney at (617) 535 4453 in order that any outstanding issues be resolved.

Applicant has included the fee for three-months extension of time fee. The Director is hereby authorized to charge any deficiency in the fees filed, asserted to be filed, or which should have been filed herewith (or with any paper hereafter filed in this application by this firm) to our

Deposit Account No. 50-1133, under Order No. 091876-0027 from which the undersigned is authorized to draw.

Respectfully submitted,

McDERMOTT WILL & EMERY LLP

/Kristin A. Connarn/

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EXAMINER	
BOGGS, RUSSELL T	

ART UNIT	PAPER NUMBER
1663	

NOTIFICATION DATE	DELIVERY MODE
10/27/2020	ELECTRONIC

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

patents.us@dentons.com

Office Action Summary	Application No. 14/112,747	Applicant(s) Lindhout et al.	
	Examiner RUSSELL T BOGGS	Art Unit 1663	AIA (FITF) Status No

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTHS FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☑ Responsive to communication(s) filed on 16 July 2020.

☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on _____.

2a)☑ This action is **FINAL.** 2b) ☐ This action is non-final.

3)☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on _____; the restriction requirement and election have been incorporated into this action.

4)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims*

5) ☑ Claim(s) 1-12,18-19,22 and 24-26 is/are pending in the application.

5a) Of the above claim(s) _____ is/are withdrawn from consideration.

6) ☐ Claim(s) _____ is/are allowed.

7) ☑ Claim(s) 1-12,18-19,22 and 24-26 is/are rejected.

8) ☐ Claim(s) _____ is/are objected to.

9) ☐ Claim(s) _____ are subject to restriction and/or election requirement

* If any claims have been determined allowable, you may be eligible to benefit from the **Patent Prosecution Highway** program at a participating intellectual property office for the corresponding application. For more information, please see http://www.uspto.gov/patents/init_events/pph/index.jsp or send an inquiry to **PPHfeedback@uspto.gov.**

Application Papers

10)☑ The specification is objected to by the Examiner.

11)☐ The drawing(s) filed on _____ is/are: a)☐ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

Priority under 35 U.S.C. § 119

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

Certified copies:

a)☐ All b)☐ Some** c)☐ None of the:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

** See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☐ Notice of References Cited (PTO-892)

2) ☐ Information Disclosure Statement(s) (PTO/SB/08a and/or PTO/SB/08b) Paper No(s)/Mail Date _____.

3) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date _____.

4) ☐ Other: _____.

DETAILED ACTION

Notice of Pre-AIA or AIA Status

The present application is being examined under the pre-AIA first-to-invent provisions.

Status

1. Claims 1-18, filed 18 October 2013, were examined and rejected in the Office action mailed 14 March 2016. Applicant responded on 14 September 2016. Claims 1-19 were examined and rejected on 15 December 2016. Applicant filed an RCE and responded to the final Office action on 15 June 2017. Claims 1-19 were examined and rejected on 22 December 2017. Applicant responded on 23 April 2018, adding claims 20-23. Claims 1-23 are examined and rejected in an Office action mailed 7 August 2018. Applicant responded on 7 February 2019 by filing an RCE; Applicant amended several claims, cancelled claims 13-17 and 21, and added claims 24-26. Claims 1-12, 18-20 and 22-26 were examined and rejected in an Office action mailed on 16 January 2020. Applicant responded on 16 July 2020, cancelling claim 20 and claim 23.

Claims 1-12, 18-19, 22, and 24-26 are examined herein.

Applicant is reminded that upon the cancellation of claims to a non-elected invention, the inventorship must be amended in compliance with 37 CFR 1.48(b) if one or more of the currently named inventors is no longer an inventor of at least one claim remaining in the application. Any amendment of inventorship must be accompanied by a request under 37 CFR 1.48(b) and by the fee required under 37 CFR 1.17(i).

Examiner's Notes

2. Citations to Applicant's specification are abbreviated herein "Spec."

Although the instant application does not claim priority to application serial no. 13/504,156 or its priority documents, many of the issues are the same except for the specifically articulated requirement for *P. infestans* resistance in the instant claim set.

In view of the lack of legibility of Figure 1 in Dr. Lindhout's declaration under 37 CFR 1.132 signed 1 May 2020, and the lack of color in the copy provided to the examiner, the examiner attempted to contact Rob Hanson and Sheila Walker separately by voicemail. No reply was received.

Withdrawal of Objections and Rejections

3. The rejection of claims 1-12, 18-19, 22, and 24-26 under 35 U.S.C. 112(b) or 35 U.S.C. 112 (pre-AIA), second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter regarding the identity of the genes recited in the claims is withdrawn in view of Applicant's amendments to the claims.

Applicant filed this application on 21 April 2011, in other words, after the 26 October 2009 filing date of application serial no. 13/504,154, now US Patent No. 10,524,436 B2, issued 7 January 2020. Many of the pending claims are therefore subject to a double patenting rejection because the specification of the '436 Patent describes that *P. infestans* resistance can be added (col. 4) to the claimed potato lines. Applicant has not filed a terminal disclaimer relative to US Patent No. 10,524,436 B2 or

application serial no. 16/541,071. Therefore the exact identity of the gene encoding resistance to *P. infestans* is still potentially important. The examiner does not take the position that adding a non-specified gene providing resistance to *P. infestans* to the deposited potato lines is not described or enabled. Insofar as the exact identity of the gene is not required by above the claims, the rejection under 35 USC 112(b) is withdrawn. The claimed potato lines are only described and enabled by the deposits, however.

4. The rejection of claims 1 and 5 under 35 U.S.C. 112(b) or 35 U.S.C. 112 (pre-AIA), second paragraph, as being indefinite regarding the use of the word "as" is withdrawn in view of Applicant's amendments to the claims.

5. The rejection of claims 1-12, 18-19, 22, and 25-26 under 35 U.S.C. 112(a) or 35 U.S.C. 112 (pre-AIA), first paragraph, as failing to comply with the enablement requirement is withdrawn in view of Applicant's amendments to the claims.

Any rejection or objection applying to a cancelled claim is rendered moot in view of the claim's cancellation.

Specification

6. The disclosure is objected to because of the following informalities.

Claim 24 recites three specific genes that are identified only by an entry in Table 10 in the specification on pages 86-87. Applicant is defining these claim terms only by reference to this table. Thus it is the Office's interpretation that it is Applicant's clear intent to incorporate the essential material cited by reference to the non-patent literature

into the claims. The meaning and scope of the claim limitations can only be understood by reference to listed references, however, such an incorporation is improper.

The incorporation of essential material into specification by reference to an unpublished U.S. application, foreign application or patent, to a publication, or to an online database is improper. Applicant is required to amend the specification, if possible, to include the material incorporated by reference, if the material is relied upon to overcome any objection, rejection, or other requirement imposed by the Office. The amendment must be accompanied by a statement executed by the applicant, or a practitioner representing the applicant, stating that the material being inserted is the material previously incorporated by reference and that the amendment contains no new matter. 37 CFR 1.57(f).

Applicant filed this application on 21 April 2011, in other words, after the 26 October 2009 filing date of application serial no. 13/504,154, now US Patent No. 10,524,436 B2, issued 7 January 2020. Many of the pending claims are therefore subject to a double patenting rejection because the specification of the '436 Patent describes that *P. infestans* resistance can be added (col. 4) to the claimed potato lines. Applicant has not filed a terminal disclaimer relative to US Patent No. 10,524,436 B2 or application serial no. 16/541,071. Therefore the exact identity of the gene encoding resistance to *P. infestans* is still potentially important. The examiner does not take the position that adding a non-specified gene providing resistance to *P. infestans* is not described or enabled. Insofar as the exact identity of the gene is not required by above the claim, the objection to the specification is maintained.

Applicant's Argument and Response

Applicant traverses the objection in the paper filed 20 July 2020. Applicant's arguments have been fully considered but are not persuasive.

Applicant refers to the recited non-patent literature referenced in the table and asserts that they are available from material deposited with CGN. Response, pp. 7-10.

In response, Applicant is aware of the deposit rules as set forth in in 37 CFR 1.801-1.809 and MPEP §§ 2402-2411.05. The cited deposits with the CGN do not meet this standard.

Table 10 also makes reference to GenBank accession numbers. The related sequences can also be brought into the sequence listing together with a statement that the sequences entered are the same sequences that were available as of the 21 April 2011 filing date of this application.

Claim Objections

7. Claims 24-25 are objected to because of the following informalities.

Claims 24-25 are objected to because they reference part (e) of claim 5 but appear to actually referring to part (f).

Appropriate correction is requested.

Claim Rejections - 35 USC § 112(b)

The following is a quotation of 35 U.S.C. 112(b):

> (B) CONCLUSION.—The specification shall conclude with one or more claims particularly pointing out and distinctly claiming the subject matter which the inventor or a joint inventor regards as the invention.

The following is a quotation of 35 U.S.C. 112 (pre-AIA), second paragraph:

> The specification shall conclude with one or more claims particularly pointing out and distinctly claiming the subject matter which the applicant regards as his invention.

8. Claims 5, 7, 19 and 24-25 are rejected under 35 U.S.C. 112(b) or 35 U.S.C. 112 (pre-AIA), second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which the inventor or a joint inventor, or for pre-AIA the applicant regards as the invention.

Claim 5 is rejected because, after amendment, part (c) reads "seeds growing a plurality of hybrid offspring potato plants from said plurality of hybrid offspring seeds." The only word left from the original part (c) is "seeds" which does not seem to have meaning in the context of the claim.

As a result, the metes and bounds of the claims are indefinite.

Dependent claims are included in this rejection because none provide limitations obviating this rejection.

Claim Rejections - 35 USC § 112(a), written description

The following is a quotation of 35 U.S.C. 112(a):

> (a) IN GENERAL.—The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same, and shall set forth the best mode contemplated by the inventor or joint inventor of carrying out the invention.

The following is a quotation of 35 U.S.C. 112 (pre-AIA), first paragraph:

> The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same and shall set forth the best mode contemplated by the inventor of carrying out his invention.

9. Claims 1-12, 18-19, 22, and 24-26 are rejected under 35 U.S.C. 112(a) or 35 U.S.C. 112 (pre-AIA), first paragraph, as failing to comply with the written description requirement. The claim(s) contains subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that

the inventor or a joint inventor, or for pre-AIA the inventor(s), at the time the application was filed, had possession of the claimed invention.

Applicant traverses the rejection in the paper filed 16 July 2020. Applicant's arguments have been fully considered but are not persuasive.

The Claims

Claim 1 is drawn to a diploid, fertile, self-compatible and essentially homozygous *S. tuberosum* potato line having an average tuber yield expressed in grams of fresh weight of at least 200 grams per plant;

wherein the self-compatibility originates from *S. chacoense* and is present in a plant of potato lines NCIMB accession number 41663, NCIMB accession number 41664, NCIMB accession number 41665, or NCIMB accession number 41765, further where representative samples were deposited with the NCIMB under breeders reference AGVD1, AGVD2, AGVD3, and AGVD17. In addition to tuber yield, the claimed line has resistance to *P. infestans* infections.

Claims 2-4 depending from claim 1 read on a hybrid potato plant or seed produced from a cross between two essentially homozygous parental breeding lines as well as a tetraploid potato plant(s) produced therefrom. Claim 8 reads on a method using a plant from claim 1. Claims 9-12 read on plants and plant parts produced by the method of claim 8. Claim 18 reads on a set of at least 2 intercrossable diploid, fertile, self-compatible and essentially homozygous *S. tuberosum* potato lines according to claim 1. Claim 22 also depends from claim 1 recites that the potato line used is a progeny of a deposited line. Dependent claim 26 requires at least 25% of the homozygous genomic loci are from the deposited lines.

Claim 5 is a method claim drawn to producing a diploid, fertile, self-compatible and essentially homozygous *S. tuberosum* potato line having an average yield of at least 200 grams per plant

starting with a first potato plant of a first diploid self-compatible, fertile and essentially homozygous potato line wherein the self-compatibility originates from *S. chacoense* and is present in a plant of potato lines NCIMB accession number 41663, NCIMB accession number 41664, NCIMB accession number 41665, or NCIMB accession number 41765, further where representative samples were deposited with the NCIMB under breeders reference AGVD1, AGVD2, AGVD3, and AGVD17

and cross-pollinating it to a second potato plant where the second potato plant has an average tuber yield expressed in grams of fresh weight of at least 200 grams per plant but any level of homozygosity;

growing a plurality of hybrid offspring potato plants;

selecting an offspring plant that is being-fertile and produces tubers at a yield of at least 200 grams per plant;

crossing said selected hybrid offspring potato plant with itself or a plant of the potato line of step (a) for between 1 to 8 generations retaining the tuber yield

while also introducing at least one trait or gene provides resistance to *Phytophthora infestans* infections.

Dependent claim 6 recites specific deposited cell lines and is not rejected under the written description requirement. Dependent claim 7 encompasses a diploid potato breeding line obtained by the method of claim 5. Claim 19 reads on an elite diploid potato breeding line obtained by the method of claim 5. Claim 24 depends from claim 5

and recites various embodiments of genes encoding *P. infestans* resistance. Claim 25 depends from claim 5 and uses transgenic means to introduce the gene or trait.

Applicant's Description

Applicant describes the deposited lines by breeders reference: AGVD1 (NCIMB 41663), AGVD2 (NCIMB 41664), AGVD3 (NCIMB 41665) and AGVD17 (NCIMB 41765). Spec., pp. 34-35.

In the specification, Applicant describes a breeding process according to the instant invention starting with a self-compatible line, e.g. AGVD1-3 or AGVD17. *Id.*, p. 39. Applicant states that "[n]o or at least little, attention is paid to the agronomical appearance of the plants at this stage." *Id.* One aspect of the recited breeding steps includes the possibility of adding a desired trait such as vigor or pest resistance. *Id.*, p. 38 & p. 37. Continuing this aspect, after adding the trait, the hybrid is backcrossed to the self-compatible line while selecting for vigor. *Id.*, p. 39. After adding numerous traits, and performing test crosses, inbred elite diploid breeding lines can be obtained which can be used to produce commercially valuable hybrids. *Id.*, pp. 44-45.

Applicant reviews the steps: develop a "a vigorous fertile homozygous self-compatible diploid potato line;" "a diploid potato breeding line;" and then "elite diploid potato breeding lines." *Id.*, p. 42. On page 44, Applicant concludes that "[t]he above method now provides a breeding process for the production of homozygous breeding lines or elite breeding lines based on the background of population AGVD1, AGVD2, AGVD3 or AGVDI7, to which background successively additional alleles are added, thereby providing elite breeding lines wherein desirable traits are stacked." *Id.*, p. 44.

In particular, in Example 1, Applicant describes obtaining the IVP07-1001/4 line from publically available germplasm where the IVP07-1001/4 plants contained the *Sli* gene after repeated selfing. Spec., p. 46. Applicant cites to the 2006 Euphytica publication by Phumichai et al. as well as the 2005 Genome publication by Phumichai et al. regarding the source of the *Sli* gene. *Id.*, p. 46 & p. 59 (*citing* to Phumichai et al. (2005) Genome 48:977-94 and Phumichai et al. (2006) Euphytica 148:227-34 (2006)). Applicant describes that these plants are of no commercial value. *Id.*, p. 46. Applicant crossed these plants to three different diploid donor lines, D1-3, in order to acquire commercially valuable traits carried by the donor lines. *Id.*, pp. 46-47. The F1 cross with D1 was selfed and the progeny deposited as AGVD1, which was estimated as 50% homozygous. *Id.*, p. 46. The F1(D1) was crossed with F1(D2) and the progeny deposited under AGVD2, and this was estimated to be 25% homozygous with respect to alleles originating from IVP07-1001/4. *Id.* The F1(D1) was backcrossed to obtain progeny 25% homozygous with respect to alleles originating from IVP07-1001/4; these were deposited as AGVD3. *Id.*, pp. 46-47.

In Example 2, Applicant describes crossing the *Sli* gene donor with various publically available potato clones designated: IVP97-079-9, IVPAA-096-I8 and SH83-92-488 to generate clones D1, D2 and D3. *Id.*, pp. 48-51. Applicant describes this created diploid trait donor lines containing the *Sli* gene with enhanced agricultural quality. *Id.* Applicant provides a more detailed description of the potato lines used in Table 6. *Id.*, pp. 59-60.

Applicant describes creating a molecular marker platform, i.e., SNPs, in Example 3, p. 51 et seq.

In Example 4, Applicant describes the development of homozygous diploid potato plants and then F1 hybrid progeny, as well as subsequent generations. *Id.*, p. 60 et seq. Applicant describes that it is "often stated" that potatoes are very prone to inbreeding depression. *Id.*, p. 62. A cause of this might be deleterious alleles; Applicant potentially identified a few such deleterious alleles, with one allele almost being lethal. *Id.*, p. 63 However, no absolute lethal allele was present. *Id., p. 62* (heading at line 30).

Applicant concludes, on pages 62-63, that

> It was found that after two generations of selfings homozygous diploid potato plants were obtained comprising alleles from both parents. Not a single marker out of 36 markers tested provided proof for a lethal allele. Furthermore, plants with more than 84% homozygous loci can be successfully crossed to obtain hybrid plants with at least five heterozygous chromosome regions. This means that hybrid seeds that are essentially uniform and that represent true potato seed have now been produced.

Id., p. 62-63.

Applicant describes the meaning of the claim term "essentially homozygous" on pages 21-22 of the instant specification and suggests that the term reflects a level of homozygosity that is above 25% or 75%. *Id.*, p. 21-22.

Applicant generally describes adding an agronomically desirable trait to a potato genotype on, e.g. page 37. In particular, the claims of this patent application require resistance to *P. infestans*.

The Scope of the Claims

The claimed genera, in particular, include those set forth in the following exemplary claims:

claim 1 – any "diploid, fertile, self-compatible and essentially homozygous *Solanum tuberosum* potato line" wherein it has a tuber yield of at least 200 g per plant and wherein the self-compatibility gene originates from *S. chacoense* and is as present in a plant of potato lines NCIMB accession number 41663, NCIMB accession number 41664, NCIMB accession number 41665, or NCIMB accession number 41765;

claim 5 – any "diploid, self-compatible and fertile potato line" wherein the self-compatibility gene originates from *S. chacoense* and is present in a plant of potato lines NCIMB accession number 41663, NCIMB accession number 41664, NCIMB accession number 41665, or NCIMB accession number 41765 as a starting material in part (a) to produce a "diploid, fertile, self-compatible and essentially homozygous *Solanum tuberosum* potato breeding line" having an average tuber yield of at least 200 grams per plant with an added trait or gene that provides resistance to *P. infestans*.

Claim 5 is a method claim but several dependent claims read on compositions produced by the method.

The above claims, however, are not limited to potato lines described by the deposits.

Claim 1 recites only tuber weight and the *Sli* gene as well as being essentially homozygous. Because claim 8 is a cross with another plant, the physiological and morphological characteristics which will be passed to any given offspring is uncertain and thus the plants produced by the method of claim 8 constitute a large genus.

Furthermore, claim 22 is drawn to any progeny line of the deposited lines. Claim 22 is unlimited with respect to the generation and thus may bear little resemblance to

the deposited lines, which, as noted above, have only minimal description associated with them. Claims 9-12 also lack any distinguishing characteristics.

The genes providing *P. infestans* resistance set forth in claim 24 are only described by Table 10. Spec., pp. 84-91. None of the listed genes are described by deposit – only by reference to an accession number and/or citation to a paper.

Case Law

The Federal Circuit court held that a written description of an invention '"requires a precise definition, such as by structure, formula [or} chemical name,' of the claimed subject matter sufficient to distinguish it from other materials." *Univ. of Cal. v. Eli Lilly & Co.*, 119 F.3d 1559, 1568, 43 USPQ2d 1398, 1405 (Fed. Cir. 1997) (*quoting Fiers v. Revel*, 984 F.2d 1164, 1171, 25 USPQ2d 1601, 1606 (Fed. Cir. 1993)). The court also stated "naming a type of material generally known to exist, in the absence of knowledge as to what that material consists of is not a description of that material." *Id.*, 119 F.3d at 1568, 43 USPQ2d at 1406. Further, the court stated that to adequately describe a claimed genus, Applicant must describe a representative number of the species of the claimed genus, and that one of skill in the art should be able to "visualize or recognize the identity of members of the genus." *Id.* The court also held that "a recitation of structural features common to members of the genus" could satisfy the written description requirement. *Id.*, 119 F.3d at 1569, 43 USPQ2d at 1406.

See also *Vas-Cath Inc. v. Mahurkar*, 19 USPQ2d 1111, 1115 (Fed. Cir., 1991), which teaches that the purpose of the written description is for the purpose of warning an innocent purchaser, or other person using a machine, of his infringement of the patent; and at the same time, of taking from the inventor the means of practicing upon

the credulity or the fears of other persons, by pretending that his invention is more than what it really is, or different from its ostensible objects, that the patentee is required to distinguish his invention in his specification. In this case, in several of the claims, such as claim 10 and claim 22 there is no way that a practitioner would be able to determine if a potentially infringing potato line is infringing the instant claims, and therefore, the public has not been put on notice with a sufficient description of the claimed invention.

Applicant Fails to Describe a Representative Number of Species or Structural Features

Applicant provides written description – by deposit – for the deposited lines – however these lines are not resistant to *P. infestans*. But does Applicant describe a representative number of species in comparison to the size of the genus claimed?

Just concerning the diploid breeding lines, Applicant only describes by deposit only four potato lines as compositions and for use in the claimed methods. All were created from the same *Sli* donor, the IVP07-1001/4 line, crossed to three diploid donor lines. Spec., p. 46. Applicant cites to Phumichai et al. (2006) as to the source of the *Sli* gene line. *Id.*. The IVP07-1001/4 line does not appear to be a *S. tuberosum* line based on a review of the specification and both Phumichai et al. (2005) and Phumichai et al. (2006), but its identity is not directly stated. Applicant does not further characterize or describe the *Sli* gene.

Similarly, Applicant fails to describe the breeding history of the deposited lines. The most detailed descriptions of D1, D2 and D3 are found in Table 6, but Table 6 fails to provide the genetic background of the lines. Spec., p. 59-60.

Additionally, Applicant fails to describe the above claimed lines that additionally have resistance to *P. infestans* with a working example.

Applicant thus fails to associate any genetic structure required for the practice of the invention.

The Art

The art describes basic the approach of the aspect relating to potato lines of the Applicant's invention. For example, in a 2009 publication, Jansky describes first obtaining homozygous diploids using a self-incompatibility inhibitor. Jansky (2009) "Breeding, Genetics, and Cultivar Development," Advances in Potato Chemistry and Technology, ed. J. Singh & L. Kaur, pp. 27-62, 46. Jansky teaches that "[h]ighly homozygous inbreds could be also be used for heterosis breeding. Jansky, p. 46. Jansky teaches that such an approach would be difficult. *Id.*

Phumichai et al. confirms the approach of Jansky, and documents the use of the *Sli* gene in the approach. Phumichai et al. (2005), abstract.

> Potato . . . is an auto- or segmental allo-tetraploid showing tetrasomic inheritance, which results in complicated genetic segregation. Since potatoes are propagated vegetatively, highly heterotic genotypes can be maintained, which facilitates the use of heterosis, an essential factor in potato breeding, particularly for tuber yield. Alternatively, open-pollinated true potato seed (TPS) can be used as a low-cost planting material for potato production.
>
> Highly homozygous inbreds, once amply produced, would be highly useful for the development of TPS varieties with genetic uniformity, as well as for the investigation of heterosis.
>
> A scheme for hybrid breeding using homozygous diploids and subsequent somatic hybridization has been proposed, for which the availability of highly homozygous diploid (or pure) lines is a key for success. In considering the use of pure lines for the development of genetically uniform [true potato seed] varieties, self-fertile, seed-propagated lines are highly desirable, and simple techniques to develop such lines are necessary.

Id., pp. 977-78 (citations omitted).

Finally, in 2016 Jansky et al. published an "Opinion & Policy" article calling for "Reinventing Potato as a Diploid Inbred Line-Based Crop." Jansky et al. (2016) Crop Sci. 56:1412-22. The authors call for the creation of "diploid inbred lines" (*id.*, p. 1413) with improved, e.g. tuber yield (*id.*, p. 1414) for use in creating inbred pools for use in heterosis. *Id.* The authors devote almost a page to the challenges faced. *Id.*, p. 1415. Then the authors addressed questions that must still be answered. *Id.* pp. 1415-18. One of the approaches cited by Jansky et al. is Dr. Lindhout's work – i.e. the instant claimed invention – which is described as being in the process of ongoing testing. *Id.*, pp. 1417-18. Therefore, approximately seven years after the filing date of the instant application, the problem of creating vigorous inbred diploid *S. tuberosum* plants apparently still existed.

Admissions in the Parent Applicant and the Art Relevant to the Scope of the Pending Claims

Aside from the resistance element, the claims read on broad genera of potato lines, but would all potato lines be active in the instant invention? What distinguishes a potato line that has 50% homozygosity and a 200 gram tuber yield from another potato line falling outside the scope of the claims?. In the specification, Applicant states that

> It is often stated that repeated selfings in diploid potato will only result in weak plants due to inbreeding depression. This should be due to numerous deleterious alleles that reduce plant fitness, when these alleles are homozygously present. Therefore, we tested the segregation of 36 markers in the six F3 populations mentioned (see Figure 5).

Spec., p. 62-63. As discussed above, Applicant summarizes the result of the study as identifying some potentially, deleterious alleles, one of which is "very" deleterious, almost lethal. *Id.*, p. 63.

The declaration by Dr. Lindhout dated 15 August 2016 in the file history of US application serial no. 13/504,156 and supporting letters document how unique the potato lines used for the instant invention are. For example, one of the letters offered in support of Dr. Lindhout's declaration by Dr. J.D.G. Jones states that

> "[I]n the mid 2000s we attempted to produce hybrid potato ourselves. . . . However, we never succeeded in developing any promising material in the form of fertile homozygotes that could be useful for hybrid breeding and that could tolerate recurrent selfing. . . . We considered that this was due to our inability to cross the Sli- self-compatibility locus into good enough or diverse enough diploid *Solanum* germplasm.

Letter from Dr. Jonathan D.G. Jones (2012), p. 1 (appended to Decl. of Dr. Lindhout 15 August 2016, ("Decl. (2016)")) (underlining added).

In the 2016 declaration, Dr. Lindhout summarizes the background art prior to the claimed invention. Decl. (2016), pp. 3-4. In particular, Dr. Lindhout states that

> That as soon as a genotype becomes homozygous for negative alleles of which the negative is not compensated by functional paralogues, such genotypes might show very poor performance and low viability;
> That inbreeding depression is manifested as a reduced fitness with a loss in both yield and fertility;
> That inbreeding depression is mainly due to the presence of many (semi) lethal alleles, and alleles with a deleterious effect, that become visible more frequently at the diploid level when the maximum number of possible alleles is reduced from four to two, resulting in homozygosity of such alleles.
> That the chance of obtaining a progeny plant, by crossing different genotypes, which is not suffering from inbreeding depression in potato is infinitesimally small, and that starting from a heterozygous diploid potato genotypes, the chance of identifying a plant without homozygous deleterious alleles is, in practice, impossible . . .

Decl. (2016), p. 4.

In a similar vein, in a paper filed with the EPO, Applicant also asserted that "every expert in the field can confirm that due to inbreeding depression, the chances of successfully attaining the plant of the invention are close to zero. Hence, the production

of such plants is generally considered impossible." EPO Publication No. EP 2 493 286 A2, Remarks to EPO (21 Dec. 2012), p. 8.

Furthermore, on 15 December 2011, approximately two years after the filing date of the 13/504,156 application, Lindhout et al. published a paper in the Potato Research journal which appears to correspond to the experiments set forth in the instant examples (all five inventors are listed as authors on the paper). Lindhout et al. (2011) Potato Res 54:301-12. The discussion in the paper is instructive. For example, Lindhout et al. describes that

> As a consequence of the high level of unfavourable alleles, techniques aimed at developing homozygotes in one step, although successful as a technology in various crops, certainly do not generate vigorous homozygous potatoes. For instance, if 20 of the 39,000 protein-encoding genes (PGSC 2011) on the potato genome harbor alleles causing severe fitness reduction, the chance to generate vigorous diploid selfed progenies without homozygous unfavourable loci from a parent genotype, that is heterozygous for these 20 loci, is only 0.3%.

Id., p. 303. Lindhout et al. concludes that "inbred lines were developed that combined self-compatibility with good agronomic performance. Although the number of such plants is still low, the progress made in this breeding programme is significant." *Id.*, p. 304. As a possible reason for success, the authors offer that "lethal alleles do not occur at high frequency in our diploid potato germplasm. *Id.*, pp. 308-09. The authors continue that

> there are many loci with minor effects involved in these traits (Quantitative Trait Locus, QTL). Our current research focuses on identifying the crucial QTLs for these traits. The results will be applied in breeding and allow to increase efficiencies by using diagnostic markers for these traits.\

Id., p. 309.

Resistance to *P. infestans*

Most of the above discussion is related to the claimed potato lines, and indeed, the specification is entitled "Hybrid Seed Potato Breeding." *Id.*, p. 1. The claims also require resistance to *P. infestans*. Claim 24, for example, recites potential sources for resistance genes.

None of the deposits are described as having the genes recited in claim 24 that encode resistance to *P. infestans*. Regarding sources of *P. infestans* resistance, Applicant provides Table 10 on pages 86-87. However, as discussed *supra*, Table 10 cannot be relied upon for essential information. Similarly, a GenBank accession number cannot be relied upon for essential information.

Applicant also describes in the response filed on 16 July several other sources for genes coding for resistance to *P. infestans*.

This aspect of the rejection only applies to claim 24. The Office agrees that a gene encoding resistance to *P. infestans* can be obtained and crossed into the claimed potato lines.

Conclusion

Applicant claims a large genus of potato lines with resistance to *P. infestans*.. Applicant fails to describe a representative number of species in comparison to the size of the genera claimed. In the instant application, Applicant describes only four potato lines to use in the claimed methods or as a starting point to obtain the claimed compositions, and all were created from the same line, the IVP07-1001/4 line. *Id.*, pp. 46-48. Applicant fails to describe working examples of the broad genus of other potato lines falling within the scope of the claims. Applicant also fails to describe any structure

necessary and/or sufficient for a potato line to be used in the claims, i.e. how to obtain *S. tuberosum* lines that are "heterozygous for a lower number of deleterious alleles" (Dr. Koornneef) or "good enough or diverse enough diploid *Solanum* germplasm" (Dr. Jones). Applicant also fails to describe other embodiments of the resistance genes as for example recited in claims 20 and 23 except by improper (*see supra*) reference to the non-patent literature and online databases.

The specification therefore fails to provide an adequate written description to support the breadth of the claims. Applicant has not demonstrated possession to one of skill in the art of the instant invention as broadly as claimed at the time of filing, and one of skill in the art would not believe Applicant to be in possession of the genus as broadly as claimed, especially in view of the letters cited above.

Dependent claims are included in this rejection because none provide further limitations obviating this rejection.

Applicant's Arguments

Applicant provides the 01 May 2020 declaration of Dr. Lindhout. Response, p. 13. Applicant cites to paragraphs 13-15 and 16-17 regarding the diversity of the deposited lines. *Id.*, pp. 13-14. Applicant then discusses the letter of Dr. Jones to Dr. Lindhout dated 16 April 2020. *Id.*, p.14. Applicant cites to paragraph 34 of the declaration. *Id.*, pp. 14-15. Paragraphs 34-35 of Dr. Lindhout's declaration deal with post-filing art with publication dates of 2016, 2017 and 2020.

Response to Applicant's Arguments

Applicant sole argument is the declaration of Dr. Lindhout. Response, pp. 15-17. The declaration under 37 CFR 1.132 filed 16 July 2020 is insufficient to overcome the

rejection of the above claims based upon 35 USC 112(a) as set forth in the last Office action because of the following reasons.

In paragraphs 8-9 of the declaration, Dr. Lindhout refers to Figure 1 of the declaration (page 4) which apparently was color-coded by Dr. Lindhout. The copy provided to the examiner of Figure 1 is not legible and is provided in black and white. (As explained *supra*, the examiner attempted to obtain a better copy.)

Applicant cites to paragraphs 10-12 of the declaration in which Dr. Lindhout describes how lines D1-D3 were created. Dr. Lindhout, in paragraph 13, asserts that these lines represent a very large genetic variation. In fact, in paragraph 14, Dr. Lindhout assets that the "genetic background of the plants of D1-D3 is thus extremely diverse." Further, "D1-D3 are not unique and do not represent any specific genotype of potato lines." *Id.* Thus, Dr. Lindhout argues "any *S. tuberosum* is equably suitable for use as a starting material in a method of the invention." Decl. 2020, para. 15.

In contrast, in application serial no. 13/504,156, Dr. Lindhout filed a declaration on 8 September 2016 to overcome a rejection under 35 USC 103 which stated in part, "[t]hat the chance of obtaining a progeny plant, by crossing different genotypes, which is not suffering from inbreeding depression in potato is infinitesimally small, and that starting from a heterozygous diploid potato genotypes, the chance of identifying a plant without homozygous deleterious alleles is, in practice, impossible. Decl. of Dr. Lindhout, app. ser. no. 13/504,156, signed 15 Aug. 2016.

Returning to the instant declaration, in paragraphs 16-17, Dr. Lindhout presents the results of an undated study which is asserted to indicate that only 4% haplotypes were unique to lines D1, D2 and D3. *Id.*, para. 17. Dr. Lindhout continues the argument

in paragraphs 18-20. Dr. Lindhout quotes from an earlier declaration submitted in US application serial no. 13/504,156.

Dr. Lindhout then turns to a letter that he received from Dr. Jones in 2020. Dr. Jones wrote in that letter that "I never made a statement to that effect, and then my words are misinterpreted."

In response, Dr. Jones' statement in his letter is perplexing. First, the letter from Dr. Jones is not submitted as a declaration itself. Second, another letter from Dr. Jones was submitted by Applicant during prosecution of application serial no. 13/504,156. This previously submitted letter from Dr. Jones is quoted in the Office action. Is Dr. Jones in 2020 repudiating that letter? Where Dr. Jones wrote that "I never made a statement to that effect, and then my words are misinterpreted," Dr. Jones' did not directly address the statements in his previous letter and there is no evidence that Dr. Jones reviewed the letter prior the comment above. The previous letter speaks for itself. How is the letter by Dr. Jones that was submitted during prosecution inaccurate? How is it misinterpreted?

In the letter attached to the 2020 Declaration, Dr. Jones also cites to personal communications (presumably with Dr. Lindhout) and a publication by Dr. Lindhout et al. The "personal communications" are not dated and the publication is post-filing art, both with respect to the instant application and the 13/504,156 application.

The Federal Circuit finds that "the test for sufficiency is whether the disclosure of the application relied upon reasonably conveys to those skilled in the art that the inventor had possession of the claimed subject matter as of the filing date. *Ariad*

Pharm., Inc. v. Eli Lilly & Co., 598 F.3d 1336, 1351, 94 U.S.P.Q.2d 1161 (Fed. Cir. 2010) (citations omitted).

Thus the quoted passages from the recent letter of Dr. Jones are not persuasive with regard to the earlier letter of Dr. Jones. Even if Dr. Jones was persuasive in contradicting his earlier letter, his new position is irrelevant to the letter from Dr. Koornneef as seen below

In support of an argument against a rejection under 35 USC 103, Applicant filed a declaration under 37 CFR 1.132 by Dr. Lindhout with supporting letters. Dr. Lindhout's declaration was formally filed in application serial no. 13/504, 156, and the supporting letters were initially filed in European Application EP2699684 (04 June 2014) and are copies therefrom. The letters teach that Applicant's invention, as broadly as claimed, was not fully described. For example, on page 2 of his letter, Dr. Koornneef states that

> Ever since Lindhout and his co-workers published their results in Potato Research volume 54 of 2011, showing high yielding hybrids from 87% homozygous F3 inbreds, I have been positively surprised that Solynta has managed to challenge this dogma. Solynta could successfully obtain essentially homozygous inbred lines by making use of genotypes that are selfcompatible and by choosing the right starting material that apparently is heterozygous for a lower number of deleterious alleles. This provides the basis of generating useful inbred lines that can become parents of F1 hybrids.

Letter from Koornneef (2012) p. 2 (underlining added). Earlier in the letter, Dr. Koornneef also states that "to start a project aiming at developing a good performing homozygous potato plant for the purpose of hybrid production is a daring undertaking with very little chance of success. *Id.*

In addition to this letter, Applicant's arguments made in response to a rejection made during European prosecution (Office action of 16 January 2020, p. 20); the quoted portions of the publication of Lindhout et al. (2011) (*id.*, pp. 20-21); and the review article by Jansky et al. (e.g. *id.*, p. 20) also support the instant rejection.

Furthermore, in application serial no. 13/504,156, Dr. Lindhout filed a declaration on 8 September 2016 to overcome a rejection under 35 USC 103 which stated in part, "[t]hat the chance of obtaining a progeny plant, by crossing different genotypes, which is not suffering from inbreeding depression in potato is infinitesimally small, and that starting from a heterozygous diploid potato genotypes, the chance of identifying a plant without homozygous deleterious alleles is, in practice, impossible. Decl. of Dr. Lindhout, app. ser. no. 13/504,156, signed 15 Aug. 2016.

Although opinion testimony must be considered, it is not controlling. MPEP § 716.01(c)(III) Thus although Dr. Lindhout and Dr. Jones assert the examiner is misinterpreting various teachings, Applicant's position is unconvincing.

The standard for a written description rejection (MPEP § 2163.04) and for considering a declaration (MPEP § 716.01(d)) is the preponderance of the evidence.. The preponderance of the evidence still requires maintaining the rejection.

Claim Rejections - 35 USC § 112(a); enablement

10. Claim 24 is rejected under 35 U.S.C. 112(a) or 35 U.S.C. 112 (pre-AIA), first paragraph, as failing to comply with the enablement requirement. The claim(s) contains subject matter which was not described in the specification in such a way as to enable

one skilled in the art to which it pertains, or with which it is most nearly connected, to make and/or use the invention.

The invention appears to employ plants or DNA from plants as a source for the genes recited in claim 24. *See* Table 10. Since the plants, or their genes, are essential to the claimed invention they must be obtainable by a repeatable method set forth in the specification or otherwise be readily available to the public. Regarding the required plants, if the plant is not so obtainable or available, the requirements of 35 USC § 112 may be satisfied by a deposit. For example, a deposit of 625 seeds of each of the claimed embodiments is considered sufficient to ensure public availability.

The specification does not disclose a repeatable process to obtain the plants and/or DNA and thus it is not apparent if the required plant and/or gene is readily available to the public.

(a) If a deposit is accepted under the terms of the Budapest Treaty, then a statement, affidavit or declaration by Applicant, or a statement by an attorney of record over his or her signature and registration number, or someone empowered to make such a statement, stating that the instant invention will be irrevocably and without restriction released to the public upon the issuance of a patent, would satisfy the deposit requirement made herein.

(b) If a deposit has not been made under the Budapest Treaty, then in order to certify that the deposit meets the criteria set forth in 37 CFR 1.801-1.809 and MPEP 2402-2411.05, Applicant may provide assurance of compliance by statement, affidavit or declaration, or by someone empowered to make the same, or by a statement by an attorney of record over his or her signature and registration number showing that:

(i) during the pendency of this application, access to the invention will be afforded to the Commissioner upon request;

(ii) all restrictions upon availability to the public will be irrevocably removed upon granting of the patent in accordance with 37 CFR § 1.808(a)(2);

(iii) the deposit will be maintained in a public depository for a period of 30 years or 5 years after the last request or for the effective life of the patent, whichever is longer;

(iv) a test of the viability of the biological material at the time of deposit (see 37 CFR § 1.807); and,

(v) the deposit will be replaced if it should ever become inviable.

In addition, the identifying information set forth in 37 CPR 1.809(d) should be added to the specification, if not already present. See 37 CPR 1.801 - 1.809 (MPEP 2401-2411.05) for additional explanation of these requirements.

Applicant does not appear to have addressed this rejection.

Claim Rejections - 35 USC § 112(a); re: Ex parte Jackson

11. Claims 1-12, 18-19, 22, and 24-26 are rejected under 35 U.S.C. 112(a) or 35 U.S.C. 112 (pre-AIA), first paragraph, because the specification, while being enabling for the deposited cell lines, does not reasonably provide enablement for forms of self-compatibility as broadly as claimed. The specification does not enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the invention commensurate in scope with these claims.

Applicant traverses the rejection in the paper filed 16 July 2020. Applicant's arguments have been fully considered but are not persuasive.

The invention appears to employ plants with novel characteristics. Since these plant characteristics are essential to the claimed invention it must be obtainable by a repeatable method set forth in the specification or otherwise be readily available to the public. If the plant is not so obtainable or available, the requirements of 35 USC § 112 may be satisfied by a deposit of the seeds. Deposits under NCIMB accession numbers 41663, 41664, 41665 and 41765 have been made. However the claims are not drawn solely to the deposits and are much broader.

The Claims

Claim 1 is drawn to a diploid, fertile, self-compatible and essentially homozygous *S. tuberosum* potato line having an average tuber yield expressed in grams of fresh weight of at least 200 grams per plant;
wherein the self-compatibility originates from *S. chacoense* and is present in a plant of potato lines NCIMB accession number 41663, NCIMB accession number 41664, NCIMB accession number 41665, or NCIMB accession number 41765, further where representative samples were deposited with the NCIMB under breeders reference AGVD1, AGVD2, AGVD3, and AGVD17. In addition to tuber yield, the claimed line has resistance to *P. infestans* infections.

Claims 2-4 depending from claim 1 read on a hybrid potato plant or seed produced from a cross between two essentially homozygous parental breeding lines as well as a tetraploid potato plant(s) produced therefrom. Claim 8 reads on a method using a plant from claim 1. Claims 9-12 read on plants and plant parts produced by the

method of claim 8. Claim 18 reads on a set of at least 2 intercrossable diploid, fertile, self-compatible and essentially homozygous *S. tuberosum* potato lines according to claim 1. Claim 22 also depends from claim 1 recites that the potato line used is a progeny of a deposited line. Dependent claim 26 requires at least 25% of the homozygous genomic loci are from the deposited lines.

Claim 5 is a method claim drawn to producing a diploid, fertile, self-compatible and essentially homozygous *S. tuberosum* potato line having an average yield of at least 200 grams per plant

starting with a first potato plant of a first diploid self-compatible, fertile and essentially homozygous potato line wherein the self-compatibility originates from *S. chacoense* and is present in a plant of potato lines NCIMB accession number 41663, NCIMB accession number 41664, NCIMB accession number 41665, or NCIMB accession number 41765, further where representative samples were deposited with the NCIMB under breeders reference AGVD1, AGVD2, AGVD3, and AGVD17

and cross-pollinating it to a second potato plant where the second potato plant has an average tuber yield expressed in grams of fresh weight of at least 200 grams per plant but any level of homozygosity;

growing a plurality of hybrid offspring potato plants;

selecting an offspring plant that is being-fertile and produces tubers at a yield of at least 200 grams per plant;

crossing said selected hybrid offspring potato plant with itself or a plant of the potato line of step (a) for between 1 to 8 generations retaining the tuber yield

while also introducing at least one trait or gene provides resistance to *Phytophthora infestans* infections.

Dependent claim 6 recites specific deposited cell lines and is not rejected under the written description requirement. Dependent claim 7 encompasses a diploid potato breeding line obtained by the method of claim 5. Claim 19 reads on an elite diploid potato breeding line obtained by the method of claim 5. Claim 24 depends from claim 5 and recites various embodiments of genes encoding *P. infestans* resistance. Claim 25 depends from claim 5 and uses transgenic means to introduce the gene or trait.

The Scope of the Claims

The claimed genera, in particular, include those set forth in the following exemplary claims:

claim 1 – any "diploid, fertile, self-compatible and essentially homozygous *Solanum tuberosum* potato line" wherein it has a tuber yield of at least 200 g per plant and wherein the self-compatibility gene originates from *S. chacoense* and is as present in a plant of potato lines NCIMB accession number 41663, NCIMB accession number 41664, NCIMB accession number 41665, or NCIMB accession number 41765;

claim 5 – any "diploid, self-compatible and fertile potato line" wherein the self-compatibility gene originates from *S. chacoense* and is present in a plant of potato lines NCIMB accession number 41663, NCIMB accession number 41664, NCIMB accession number 41665, or NCIMB accession number 41765 as a starting material in part (a) to produce a "diploid, fertile, self-compatible and essentially homozygous *Solanum tuberosum* potato breeding line" having an average tuber yield of at least 200 grams per plant with an added trait or gene that provides resistance to *P. infestans*.

Claim 5 is a method claim but several dependent claims read on compositions produced by the method.

The above claims, however, are not limited to potato lines described by the deposits.

Claim 1 recites only tuber weight and the *Sli* gene as well as being essentially homozygous. Because claim 8 is a cross with another plant, the physiological and morphological characteristics which will be passed to any given offspring is uncertain and thus the plants produced by the method of claim 8 constitute a large genus.

Furthermore, claim 22 is drawn to any progeny line of the deposited lines. Claim 22 is unlimited with respect to the generation and thus may bear little resemblance to the deposited lines, which, as noted above, have only minimal description associated with them. Claims 9-12 also lack any distinguishing characteristics.

The genes providing *P. infestans* resistance set forth in claim 24 are only taught by Table 10. Spec., pp. 84-91. None of the listed genes are described by deposit – only by reference to an accession number and/or citation to a paper.

In re Wands

The Federal Circuit in *In re Wands* lists eight considerations for determining whether or not undue experimentation would be necessary to practice an invention. *In re Wands*, 858 F2d 731, 737, 8 USPQ2d 1400, 1404 (Fed. Cir. 1988). These factors are: the quantity of experimentation necessary, the amount of direction or guidance presented, the presence or absence of working examples of the invention, the nature of the invention, the state of the prior art, the relative skill of those in the art, the predictability or unpredictability of the art, and the breadth of the claims. *Id.*

Applicant's Teachings

In the specification, Applicant teaches obtaining the IVP07-1001/4 line from publically available germplasm and plants that contain the *Sli* gene and using these plants to breed AGVD1, AGVD2, and AGVD3. Spec., pp. 46-47. Applicant teaches that lines were deposited and that the deposited lines are known by their breeders reference: AGVD1 (NCIMB 41663), AGVD2 (NCIMB 41664), AGVD3 (NCIMB 41665) and AGVD17 (NCIMB 41765). *Id.*, pp. 34-35. Applicant teaches a breeding process according to the instant invention starting with AGVD1-3 or AGVD17 on page 39 et seq.

Applicant teaches crossing the *Sli* gene donor with various potato clones designated IVP97-079-9, IVPAA-096-I8 and SH83-92-488 to generate clones D1, D2 and D3. *Id.*, p. 49. Applicant teaches creating a line containing the *Sli* gene with enhanced agricultural quality in Example 2, pp. 48-51. Applicant teaches creating a molecular marker platform, i.e., SNPs, in Example 3, p. 51 et seq. In Example 4, Applicant teaches the development of homozygous diploid potato plants and then F1 hybrid progeny, as well as subsequent generations. *Id.*, p. 60 et seq. Applicant teaches the meaning of the claim term "essentially homozygous" on pages 21-22 of the instant specification and suggests that the term reflects a level of homozygosity that is above 25% or 75%. *Id.*, p. 21-22. Applicant generally teaches adding an agronomically desirable trait to a potato genotype on, e.g. page 37, which includes resistance to *P. infestans*.

The Art

The art generally teaches this breeding strategy, or similar strategies for producing the claimed lines aside from the resistance. For example, Jansky teaches

first obtaining homozygous diploids using a self-incompatibility inhibitor. Jansky (2009) "Breeding, Genetics, and Cultivar Development," Advances in Potato Chemistry and Technology, ed. J. Singh & L. Kaur, pp. 27-62, 46. Jansky teaches that "[h]ighly homozygous inbreds could be also be used for heterosis breeding. *Id.* Jansky teaches that such an approach would be difficult. *Id.*

Phumichai et al. confirms the approach of Jansky, and documents the use of the *Sli* gene in the approach. Phumichai et al. (2005) Genome 48:977-84, abstract.

> Potato (Solanum tuberosum) is an auto- [sic] or segmental allo-tetraploid showing tetrasomic inheritance, which results in complicated genetic segregation. Since potatoes are propagated vegetatively, highly heterotic genotypes can be maintained, which facilitates the use of heterosis, an essential factor in potato breeding, particularly for tuber yield. Alternatively, open-pollinated true potato seed (TPS) can be used as a low-cost planting material for potato production.
>
> Highly homozygous inbreds, once amply produced, would be highly useful for the development of TPS varieties with genetic uniformity, as well as for the investigation of heterosis."
>
> "A scheme for hybrid breeding using homozygous diploids and subsequent somatic hybridization has been proposed, for which the availability of highly homozygous diploid (or pure) lines is a key for success. In considering the use of pure lines for the development of genetically uniform [true potato seed] varieties, self-fertile, seed-propagated lines are highly desirable, and simple techniques to develop such lines are necessary." .

Id., pp. 977-78 (citations omitted).

Admissions in the Parent Applicant and the Art Relevant to the Scope of the Pending Claims

In the response filed on 14 September 2016 ("Sept. Response"), Applicant traverses the rejection of record at that time under 35 USC 103 and argues that one of skill in the art would not have a reasonable expectation of success in obtaining lines such as those deposited. 14 Sept. 2016 Response, pp. 16-22. As part of this

argument, Applicant reviews the scope and content of the prior art, including the references cited above. For example, Applicant cites to a publication from Watanabe et al. (Breed Sci 46:329-36 (1996)) as standing for "homozygous inbred lines have not been obtained at the diploid level due to both inbreeding depression and self-incompatibility." Sept. Response, p. 17. Applicant also cites to Jansky as downplaying the possibility of hybrid breeding as 'commercially unfeasible due to "dramatic reductions in fertility and vigor.'" Sept. Response, p. 17 (quoting Jansky, p. 46). Applicant also argues that Uijtewaal et al. (Euphytica 36: 745-753, (1987)) confirms this teaching. Response, p. 17 & p. 19. Applicant concludes the argument with citation of additional material supporting their position that one of skill in the art would not have a reasonable expectation of success in combining the art cited in the rejection under 35 USC 103.

In support of the argument, Applicant also filed a declaration under 37 CFR 1.132 by Dr. Lindhout with supporting letters. Dr. Lindhout's declaration was formally filed in application serial no. 13/504,156, and the supporting letters were initially filed in European Application EP2699684 (04 June 2014) and are copies therefrom. The letters teach that the success of Applicant's invention, as broadly as claimed, was unpredictable. For example, on page 2 of his letter, Dr. Koornneef states that

> Ever since Lindhout and his co-workers published their results in Potato Research volume 54 of 2011, showing high yielding hybrids from 87% homozygous F3 inbreds, I have been positively surprised that Solynta has managed to challenge this dogma. Solynta could successfully obtain essentially homozygous inbred lines by making use of genotypes that are selfcompatible and by choosing the right starting material that apparently is heterozygous for a lower number of deleterious alleles. This provides the basis of generating useful inbred lines that can become parents of F1 hybrids.

Letter from Koornneef (2012) p. 2 (underlining added). Earlier, Dr. Koornneef also states that "to start a project aiming at developing a good performing homozygous potato plant for the purpose of hybrid production is a daring undertaking with very little chance of success. *Id.*

In another letter supplied in support of Dr. Lindhout's declaration, Dr. Jones states that

> "[I]n the mid 2000s we attempted to produce hybrid potato ourselves. . . . However, we never succeeded in developing any promising material in the form of fertile homozygotes that could be useful for hybrid breeding and that could tolerate recurrent selfing. . . . We considered that this was due to our inability to cross the Sli- self-compatibility locus into good enough or diverse enough diploid *Solanum* germplasm.

Letter from Dr. Jonathan D.G. Jones (2012), p. 1 (underlining added).

Applicant claims a large genus of potato plants / lines. Jansky and Spooner et al. describes the breadth of the of the claimed genus as extremely large even without considering resistance. See, e.g., Jansky (2009), pp. 27-29; Spooner et al. (2014) Bot Rev 80:283-383, 286-94 (Table 1). Applicant's claimed invention reads on any potential potato line that is a "diploid, fertile, self-compatible and essentially homozygous *Solanum tuberosum* potato line" with a tuber yield of 200 g where the *Sli* gene is in the deposited lines.

In view of Applicant's arguments in response to the rejection under 35 USC 103 and Dr. Lindhout's supporting letters, success in practicing Applicant's claimed invention would be unpredictable and would depend on the genotype of the potato lines which would be required to add resistance to *P. infestans*. Therefore, extensive guidance would be required to enable the invention as broadly as claimed. Such guidance is not provided.

The specification therefore does not enable one skilled in the art to make and use members of the species of the claimed genus other than the deposited lines. See, for example, MPEP 2164.06(a)(II), citing to *Ex parte Jackson*, 217 USPQ 804, 806 (Bd. App. 1982). The issue before the Board in *Ex parte Jackson* was:

> The issue squarely raised by this rejection is whether or not a description of several newly discovered strains of bacteria having one particularly desirable metabolic property in terms of the conventionally measured culture characteristics and a number of metabolic and physiological properties would enable one of ordinary skill in the relevant art to independently discover additional strains having the same specific, desirable metabolic property, i.e., the production of a particular antibiotic.
>
> To state the issue somewhat differently, it is whether a verbal description of a new species would enable one of ordinary skill in the relevant art to obtain strains of that species over and above the specific strains made available through deposit in one of the recognized culture depositories.

Ex parte Jackson, 217 USPQ 804, 806 (Bd. App. 1982). In the instant application, the issue is similar to the one before the Board in that Applicant made deposits with unique characteristics but not taught how to identify other lines. In *Ex parte Jackson*, the Board held that the deposit, without a detailed explanation of how to obtain more species from nature, did not enable the generic claims. *Id.*, p. 808. The board found that "the degree of experimentation involved in locating new [organisms] apart from deposited cultures is undue in light of the enablement requirement of 35 U.S.C. 112." *Id.* The *Jackson* decision was considered and distinguished by the Federal Circuit in the *Wands* decision in view of the routine experimentation required to obtain hybridomas. *In re Wands*, 858 F.2d at 737-37, 8 USPQ at 1403-04.

The claimed but not enabled genera also include genes providing resistance to *P. infestans* as listed in claim 24 where the genes also listed in Table 10. The entries in

Table 10, insofar as the provide matter essential to determining the scope of the claims, are not enabled.

Conclusion

Applicant fails to enable the invention as broadly claimed. For example, the Supreme Court held that "[h]he basic quid pro quo contemplated by the Constitution and the Congress for granting a patent monopoly is the benefit derived by the public from an invention with substantial utility. Unless and until a process is refined and developed to this point--where specific benefit exists in currently available form--there is insufficient justification for permitting an applicant to engross what may prove to be a broad field." *Brenner v. Manson*, 383 U.S. 519, 148 USPQ 689, 695 (1966). Applicant teaches only four embodiments without resistance. In view of, for example, the surprise of Dr. Koornneef and Dr. Jones, Applicant fails to provide the public with the knowledge of how to practice Applicant's invention with regard to just the potato lines with the exception of the embodiments of the deposited lines.

Therefore, in view of the *Ex parte Jackson* decision, the generic scope of, e.g. claims 1 and 5, reading on the genera discussed above, is not enabled. Obtaining further species falling within the scope of the generic claim would require undue experimentation.

Dependent claims are included in this rejection because none provide further limitations obviating this rejection.

Applicant's Arguments

Applicant traverses the rejection on pages 15-17 of the response. Applicant's sole argument is based on the declaration of Dr. Lindhout signed on 01 May 2020.

Response, p. 15. Applicant cites to paragraphs 13-15 and 16-17 regarding the diversity of the deposited lines. *Id.*, pp. 15-16. Applicant then discusses the letter of Dr. Jones to Dr. Lindhout dated 16 April 2020. *Id.*, p.16-17.

Response to Applicant's Arguments

Applicant sole argument is the declaration of Dr. Lindhout. Response, pp. 15-17. The declaration under 37 CFR 1.132 filed 16 July 2020 is insufficient to overcome the rejection of the above claims based upon 35 USC 112(a) as set forth in the last Office action because of the following reasons.

In paragraphs 8-9 of the declaration, Dr. Lindhout refers to Figure 1 of the declaration (page 4) which apparently was color-coded by Dr. Lindhout. The copy provided to the examiner of Figure 1 is (1) not legible and (2) is provided in black and white. (As seen *supra*, the examiner attempted to obtain a better copy.)

Applicant cites to paragraphs 10-12 of the declaration in which Dr. Lindhout describes how lines D1-D3 were created. Dr. Lindhout, in paragraph 13, asserts that these lines represent a very large genetic variation. In fact, in paragraph 14, Dr. Lindhout assets that the "genetic background of the plants of D1-D3 is thus extremely diverse." Further, "D1-D3 are not unique and do not represent any specific genotype of potato lines." Thus, "any *S. tuberosum* is equably suitable for use as a starting material in a method of the invention." Decl. 2020, para. 15.

In contrast, in application serial no. 13/504,156, Dr. Lindhout filed a declaration on 8 September 2016 to overcome a rejection under 35 USC 103 which stated in part, "[t]hat the chance of obtaining a progeny plant, by crossing different genotypes, which is not suffering from inbreeding depression in potato is infinitesimally small, and that

starting from a heterozygous diploid potato genotypes, the chance of identifying a plant without homozygous deleterious alleles is, in practice, impossible. Decl. of Dr. Lindhout, app. ser. no. 13/504,156, signed 15 Aug. 2016.

Returning to the instant declaration, in paragraphs 16-17, Dr. Lindhout presents the results of an undated study which is asserted to indicate that only 4% haplotypes were unique to lines D1, D2 and D3. *Id.*, para. 17. Dr. Lindhout continues the argument in paragraphs 18-20. Dr. Lindhout quotes from an earlier declaration submitted in US application serial no. 13/504,156.

Dr. Lindhout then turns to a letter that he received from Dr. Jones. Dr. Jones wrote that "I never made a statement to that effect, and then my words are misinterpreted."

In response, Dr. Jones' statement in his letter is perplexing. First, the letter from Dr. Jones is not submitted as a declaration itself. Second, a letter from Dr. Jones was submitted by Applicant during prosecution. This letter from Dr. Jones is quoted in the Office action. Is Dr. Jones to repudiating that letter? Where Dr. Jones wrote that "I never made a statement to that effect, and then my words are misinterpreted," Dr. Jones' did not directly address the statements in his previous letter and there is no evidence that Dr. Jones reviewed the letter prior the comment above. The previous letter speaks for itself. How is the letter by Dr. Jones that was submitted during prosecution inaccurate? How is it misinterpreted?

In the letter attached to the 2020 Declaration, Dr. Jones also cites to personal communications (presumably with Dr. Lindhout) and a publication by Dr. Lindhout et al.

The "personal communications" are not dated and the publication is post-filing art, both with respect to the instant application and the 13/504,156 application.

The MPEP requires that the specification must be enabling as of the filing date. MPEP § 2164.05(a).

Thus the quoted passages from the recent letter of Dr. Jones are not persuasive with regard to the earlier letter of Dr. Jones. Even if Dr. Jones was persuasive in contradicting his earlier letter, his new position is irrelevant to the letter from Dr. Koornneef (Office action of 16 January 2020, p. 33), Applicant's arguments made in response to a rejection made during European prosecution (*id.*, p. 20); the quoted portions of the publication of Lindhout et al. (2011) (*id.*, pp. 20-21); and the review article by Jansky et al. (*id.*, p. 20).

Although opinion testimony provided in a declaration must be considered, it is not controlling. MPEP § 716.01(c)(III) Thus although Dr. Lindhout and Dr. Jones assert the examiner is misinterpreting various teachings, Applicant's position is unconvincing.

The general standard for a *prima facie* rejection and for considering a declaration (MPEP § 716.01(d)) is the preponderance of the evidence. E.g., 37 CFR 1.56(b). The preponderance of the evidence still requires maintaining the rejection.

Double Patenting

The nonstatutory double patenting rejection is based on a judicially created doctrine grounded in public policy (a policy reflected in the statute) so as to prevent the unjustified or improper timewise extension of the "right to exclude" granted by a patent and to prevent possible harassment by multiple assignees. A nonstatutory obviousness-type double patenting rejection is appropriate where the conflicting claims are not identical, but at least one examined application claim is not patentably distinct from the reference claim(s) because the examined application claim is either anticipated by, or would have been obvious over, the reference claim(s). See, e.g., *In re Berg*, 140

F.3d 1428, 46 USPQ2d 1226 (Fed. Cir. 1998); *In re Goodman*, 11 F.3d 1046, 29 USPQ2d 2010 (Fed. Cir. 1993); *In re Longi*, 759 F.2d 887, 225 USPQ 645 (Fed. Cir. 1985); *In re Van Ornum*, 686 F.2d 937, 214 USPQ 761 (CCPA 1982); *In re Vogel*, 422 F.2d 438, 164 USPQ 619 (CCPA 1970); and *In re Thorington*, 418 F.2d 528, 163 USPQ 644 (CCPA 1969).

A timely filed terminal disclaimer in compliance with 37 CFR 1.321(c) or 1.321(d) may be used to overcome an actual or provisional rejection based on a nonstatutory double patenting ground provided the conflicting application or patent either is shown to be commonly owned with this application, or claims an invention made as a result of activities undertaken within the scope of a joint research agreement.
Effective January 1, 1994, a registered attorney or agent of record may sign a terminal disclaimer. A terminal disclaimer signed by the assignee must fully comply with 37 CFR 3.73(b).

The USPTO internet Web site contains terminal disclaimer forms which may be used. Please visit http://www.uspto.gov/forms/. The filing date of the application will determine what form should be used. A web-based eTerminal Disclaimer may be filled out completely online using web-screens. An eTerminal Disclaimer that meets all requirements is auto-processed and approved immediately upon submission. For more information about eTerminal Disclaimers, refer to http://www.uspto.gov/patents/process/file/efs/guidance/eTD-info-I.jsp.

12. Claims 1-12, 18-19, 22 and 25-26 are provisionally rejected on the ground of nonstatutory obviousness-type double patenting as being unpatentable over claims 35-48 of copending Application No. 16/541,071 (the '071 Application). Although the conflicting claims are not identical, they are not patentably distinct from each other. This is a provisional double patenting rejection since the conflicting claims have not yet been patented.

The instant claimed potato lines are based on the same deposits as the '071 Application. The specification of the '071 Application also teaches that *P. infestans* resistance can be added (p. 6 of the '071 Application's specification). Therefore the above instant claims are obvious variations of the patented claims.

Applicant did not argue against the rejection (Response, p. 17) and the rejection is maintained.

13. Claims 1-12, 18-19, 22 and 25-26 are rejected on the ground of nonstatutory double patenting as being unpatentable over claims 1-19 of U.S. Patent No. 10,524,436 (the '436 Patent). Although the claims at issue are not identical, they are not patentably distinct from each other because of the reasons as follows.

The instant claimed potato lines are based on the same deposits as the '436 Patent. The specification also teaches that *P. infestans* resistance can be added (col. 4). Therefore the above instant claims are obvious variations of the patented claims.

Applicant did not argue against the rejection (Response, p. 17) and the rejection is maintained.

Conclusion

14. No claim is allowed.

Applicant's amendment necessitated the new ground(s) of rejection presented in this Office action. Accordingly, **THIS ACTION IS MADE FINAL**. See MPEP § 706.07(a). Applicant is reminded of the extension of time policy as set forth in 37 CFR 1.136(a).

A shortened statutory period for reply to this final action is set to expire THREE MONTHS from the mailing date of this action. In the event a first reply is filed within TWO MONTHS of the mailing date of this final action and the advisory action is not

mailed until after the end of the THREE-MONTH shortened statutory period, then the shortened statutory period will expire on the date the advisory action is mailed, and any extension fee pursuant to 37 CFR 1.136(a) will be calculated from the mailing date of the advisory action. In no event, however, will the statutory period for reply expire later than SIX MONTHS from the date of this final action.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to RUSSELL T BOGGS whose telephone number is (571)272-2805. The examiner can normally be reached on Monday - Friday, 0800 to 1830 Mtn.

Examiner interviews are available via telephone, in-person, and video conferencing using a USPTO supplied web-based collaboration tool. To schedule an interview, applicant is encouraged to use the USPTO Automated Interview Request (AIR) at http://www.uspto.gov/interviewpractice.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Shubo (Joe) Zhou, can be reached on 571-272-0724. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see https://ppair-my.uspto.gov/pair/PrivatePair. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free). If you would like assistance from a USPTO Customer Service Representative or access to the automated information system, call 800-786-9199 (IN USA OR CANADA) or 571-272-1000.

/RUSSELL T BOGGS/
Examiner, Art Unit 1663

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Sequence of the structural gene for granule-bound starch synthase of potato (*Solanum tuberosum* L.) and evidence for a single point deletion in the *amf* allele

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Summary. The genomic sequence of the potato gene for starch granule-bound starch synthase (GBSS; "waxy protein") has been determined for the wild-type allele of a monoploid genotype from which an amylose-free (amf) mutant was derived, and for the mutant part of the *amf* allele. Comparison of the wild-type sequence with a cDNA sequence from the literature and a newly isolated cDNA revealed the presence of 13 introns, the first of which is located in the untranslated leader. The promoter contains a G-box-like sequence. The deduced amino acid sequence of the precursor of GBSS shows a high degree of identity with monocot waxy protein sequences in the region corresponding to the mature form of the enzyme. The transit peptide of 77 amino acids, required for routing of the precursor to the plastids, shows much less identity with the transit peptides of the other waxy preproteins, but resembles the hydropathic distributions of these peptides. Alignment of the amino acid sequences of the four mature starch synthases with the *Escherichia coli glgA* gene product revealed the presence of at least three conserved boxes; there is no homology with previously proposed starch-binding domains of other enzymes involved in starch metabolism. We report the use of chimeric constructs with wild-type and *amf* sequences to localize, via complementation experiments, the region of the *amf* allele in which the mutation resides. Direct sequencing of polymerase chain reaction products confirmed that the *amf* mutation is a deletion of a single AT basepair in the region coding for the transit peptide. Premature termination of translation as a result of this frameshift mutation results in a small peptide. However, a protein reacting with anti-GBSS serum, slightly larger than the wild-type mature GBSS, can be detected in a membrane fraction from amylose-free tubers. A possible explanation for this phenomenon will be discussed.

Key words: Frameshift – Potato – Starch – Transit peptide – Waxy

Offprint requests to: F.R. van der Leij

Introduction

Granule-bound starch synthase (GBSS, starch granule-bound ADP(UDP)glucose:α-1,4-D-glucan 4-α-glucosyltransferase, EC 2.4.1.21), also called the waxy protein (Echt and Schwartz 1981), is a nuclear-encoded enzyme of about 60 kDa which is active in starch synthesizing plastids of plants (Shannon and Garwood 1984; Vos-Scheperkeuter et al. 1986). Although GBSS catalyses in vitro the elongation of both amylose and amylopectin (Leloir et al. 1961), mutations affecting GBSS genes in several plant species result in a loss of amylose only, while the total amounts of starch remain the same (Shannon and Garwood 1984). Recently, Smith (1990) has provided evidence that in pea the waxy protein is not the major starch granule-bound starch synthase. In this plant species a protein of 77 kDa, which can be solubilized from starch granules and which is antigenically only weakly related to the waxy protein of potato, seems to be the major GBSS. However, it is not known whether this enzyme is responsible for amylose synthesis.

Application of the iodine stain for starch to pollen and maize endosperm has made the waxy character one of the most useful characters for genetic studies in plants. After the isolation of the *Waxy* gene from maize (Shure et al. 1983), the sequences of the structural genes from maize (Klösgen et al. 1986), barley (Rohde et al. 1988) and rice (Wang et al. 1990) have been determined, and also partial sequences from the potato gene have been published (Hergersberg 1988; Rohde et al. 1990). In this study we present the complete sequence of the potato GBSS gene. We compare the amino acid sequence deduced from the potato gene with those deduced from the *Waxy* genes from maize, barley and rice and from the gene for glycogen synthase from *Escherichia coli*.

We also present an analysis of the GBSS gene from an amylose-free (amf) mutant of potato. This monogenic recessive mutant was isolated after X-irradiation (Hovenkamp-Hermelink et al. 1987) and lacks amylose in all starch-containing tissues (Jacobsen et al. 1989). Analogous to *waxy* mutations in maize and rice (Wessler and

Varagona 1985; Okagaki and Wessler 1988), the *amf* mutation is located in the structural gene for GBSS. This was shown in a complementation experiment using *Agrobacterium rhizogenes*-mediated transfer of binary constructs containing the wild-type genomic GBSS sequence into the mutant (van der Leij et al. 1991). Subsequent analysis of transformed hairy roots and regenerants showed the presence of active GBSS and amylose in the starch of the complemented mutants. Therefore, in potato, GBSS is the sole enzyme responsible for the presence of amylose in all starch-containing tissues.

Earlier restriction pattern comparison between cloned GBSS genes from the wild-type parent potato clone AM79.7322 (2n=x=12) and the amf mutant 86.040 (2n=x=12) revealed no detectable differences (Visser et al. 1989). The same result was obtained after comparison of the wild-type allele with an *amf* allele isolated from an amylose-free F_2 plant (this report). The presence of a normally sized GBSS mRNA in amylose-free plants indicated that the mutation had no negative effect on the efficiency of transcription (Visser et al. 1989). Therefore, we expected the *amf* mutation to be a small lesion in the transcribed part of the GBSS gene. Here we provide evidence that the *amf* mutation causes a shift in the reading frame which leads to the subsequent termination of translation in the region coding for the transit peptide, the part of the pre-protein needed for efficient transport across the plastid membrane. We deduced the post-translational processing site of the wild-type pre-GBSS by comparison with the N-terminus of a mature GBSS from potato and discuss the putative (re-)initiation of translation in the amf mutant which results in production of an inactive GBSS-like protein.

Materials and methods

Plant material. Clones obtained from homozygous amf plants 1029-32 and 1031-29 (2n=2x=24) from families of $F_1 \times F_1$ crosses (Jacobsen et al. 1989) and the diploidized (2n=2x=24) original mutant 86040 were grown in vitro as previously described (van der Leij et al. 1991). For the construction of libraries, cultivar (cv.) Maris Piper (2n=4x=48) and 1031-29 plants were grown in a greenhouse. Tubers from cv. Doré were used for the isolation of wild-type GBSS protein.

Standard methods and reagents. Manipulations of DNA were performed essentially as described in Maniatis et al. (1982), plasmid isolations were according to Birnboim and Doly (1979). Recombinant plasmids were purified by means of PEG precipitation (0.4 M NaCl, 12.5% polyethyleneglycol 6000, 1 h, 0° C) and extensive washing with 80% ethanol for sequencing by the dideoxy chain termination method (Sanger et al. 1977).

Sequencing of the wild-type GBSS gene. The lambda EMBL4 clone $LGBSS^{wt}$-6 (Visser et al. 1989) was subcloned in pUC18 (Yanisch-Perron et al. 1985), using the *E. coli* K12 strain JM83 (Vieira and Messing 1982) as host. A combination of sequencing subclones and unidirectional deletion clones and primer walking was used to determine the sequences of both strands of the three *Hin*dIII fragments, except for a part of DNA further than 380 bp downstream of the transcribed region, which was sequenced in one direction only (Fig. 4A).

A lambda gt11 potato cDNA library was constructed with the Amersham cDNA cloning kit, using tubers of cv. Maris Piper as source for RNA. Screening of 120000 plaques yielded 30 positive clones after hybridization with the GBSS cDNA isolated earlier from cv. Granola (Hergersberg 1988). Ten of the positive clones were further characterized. One clone hybridized with the 5′ *Eco*RI fragment of the previously isolated cDNA and was subcloned for sequencing.

Isolation of GBSS protein and sequencing of the N-terminus. Proteins were extracted from potato starch granules as described by Vos-Scheperkeuter et al. (1986). The protein extract was separated on an 8% SDS-polyacrylamide gel, transferred as described by Hovenkamp-Hermelink et al. (1987) to polyvinylidene difluoride (PVDF) membrane (Millipore) and stained with Coomassie Brilliant Blue as described by Matsudaira (1987). Approximately 15 µg of GBSS was excised from the filter and sequenced by automatic Edman degradation (Sequenator 477A, Applied Biosystems). Membrane fractions were isolated from potato tubers as described previously by Lavintman et al. (1974).

Cloning of the amf *allele.* A genomic bank of the amf plant 1031-29 was constructed with *Bam*HI arms of lambda GEM11 (Promega). From 230000 plaques, three positive recombinants were picked up, one hybridizing with probes upstream and downstream of the three *Hin*dIII fragments carrying the gene. This clone, designated G1107, does not contain the downstream *Bgl*II site, which enables asymmetrical subcloning using *Bgl*II and *Sal*I, as lambda GEM11 has a site for *Sal*I near the *Bam*HI site (Frischauf et al. 1983). The *amf* allele was subcloned as a *Bgl*II-*Sal*I fragment in pMTL20 (Chambers et al. 1988), yielding the construct pAMF210. The same fragment from G1107 was ligated between the *Bam*HI and *Sal*I sites of the plant transformation vector pBIN19 (Bevan 1984) to give pAMF110 (Fig. 4C).

Construction of chimeric genes. The binary plasmid pAMF110 was used as the backbone for the construction of pWM114 (Fig. 4C). For replacement of the *Eco*RI fragment carrying the 5′ part of the gene, pAMF110 was cut with *Eco*RI, religated and transformed into *E. coli.* The resulting plasmid, pAMF112, was linearized with *Eco*RI and treated with calf intestinal alkaline phosphatase, before insertion of the corresponding wild-type fragment. Plasmid pMW104 (Fig. 4C) was made the other way round, with pWAM100 (van der Leij et al. 1991) as backbone and pAMF210 as source of the mutant gene fragment.

Removal of the upstream *Hin*dIII site in pWAM10, which contains the wild-type 1.2 kb promoter and 5′ coding region was done by a Klenow reaction on a par-

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