

General discussion

Introduction

The research described in this thesis comprises the isolation of a potato late blight resistance gene, the understanding of the genomic organization and evolution of the major late blight resistance complex in potato, and the probing of the natural mechanisms adopted by wild species. This study contributes to the ultimate goal of sustainable disease management of late blight in potato, a key issue for agriculture in China, the Netherlands, and in fact worldwide.

in vitro Inoculation-a Household Tool

Genetic characterization of *R* genes in hosts and *Avr* genes in pathogens requires an efficient method of disease testing. Several methods have been developed for the potato-*P. infestans* pathosystem. These include field tests and whole plant greenhouse assays (Stewart *et al.*, 1983), laboratory tests on detached leaves (Lapwood, 1961), leaflets (Malcolmson, 1969) or leaf discs (Hodgson, 1961). Vleeshouwers et al. (1999) systematically compared these methods and concluded that the detached leaf assay is a good alternative to field tests.

The *in vitro* inoculation method we developed (**Chapter 2**) is a good alternative to the detached leaf assay, particularly when major *R-Avr* interactions are investigated. In our lab, we apply it for multiple purposes: disease testing of segregating populations or of primary transformants, screening for new resistance sources from a large collection of wild germplasms, and avirulence profiling of *P. infestans* isolates. It is a convenient household tool, since one can plan the inoculation experiments more flexibly, without concern about greenhouse space and seasonal variation. Important plants can be easily multiplied and rechecked with different isolates afterwards.

R Gene Cloning in Potato

Potato, the most important non-cereal crop, suffers from many diseases and pests caused by a diversity of bacterial, fungal, oomycete, and viral pathogens as well as nematodes and insects. The yield loss caused by diseases and pests in potato is estimated at 22% per year (Ross, 1986). Plant disease resistance, often conferred by single dominant resistance (*R*) genes, is a very important agronomic trait. The isolation of *R* genes is a prerequisite to understanding their role in pathogen surveillance and to a better deployment of genetic resistance for disease control.

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A number of *R* genes in various plants have been isolated using virtually three strategies, transposon tagging, map-based cloning, and candidate gene approach. Transposon tagging is hardly applicable in potato. Firstly, it is difficult to develop a diploid potato clone carrying a transposon near the *R* locus of interest. Secondly, homozygous resistant plants needed for transposon tagging are mostly lethal. In an *R3r3* x *R3r3* cross, El Kharbotly (*unpublished work*) found no *R3R3* progeny by testcrossing with an *r3r3* clone. Map-based cloning was successful in isolating several *R* genes from potato (Bendahmane et al., 1999; van der Vossen et al., 2000; Ballvora et al., 2002; Song et al., 2003; van der Vossen et al., 2003; Paal et al., 2004, and **Chapter 4**). The candidate gene approach was successful in the case of cloning of the *Rx2* gene for PVX extreme resistance (Bendahmane *et al.*, 2000). With more knowledge of the genomic organization of *R* loci, the candidate gene approach will play a more prominant role in *R* gene isolation. However, most *R* genes reside in complex loci where dynamic evolution has occurred (Michelmore and Meyers, 1998; Hulbert et al., 2001), resulting in considerable intra- and interspecific polymorphisms at *R* loci. The candidate gene approach can only play a supportive role in map-based cloning.

Even in the era of genomics, map-based cloning of *R* genes from crop species such as potato is far from a routine procedure. Most *R* genes are introgressed from wild species and they are characterized in the background of cultivated species. Dynamic evolution resulted in large sequence divergence between the 'wild' resistant haplotype and the 'domestic' susceptible haplotype. The implication of this divergence for map-based cloning is two fold. On one side, saturation of markers at the *R* gene region can be achieved. In the potato UHD map, we found 43 AFLP markers cosegregating with the *R3* locus. On the other side, recombination is seriously inhibited. The estimated physical/genetic distance ratio is at least 10 Mb/cM in the *R3a* region. The extremely low recombination frequency makes chromosome walking an indispensable step to make a physical contig for the target region. Chromosome walking at complex *R* loci is a very time-consuming process due to their highly repetitive nature. Transposable elements were often found at the ends of BACs derived from the *R3a* region, which made extension of contigs impossible. In potato, this process is further complicated by its heterozygous genome. Often chromosome walking goes to the *r* haplotypes (Kanyuka *et al.*, 1999).

To partially circumvent these obstacles, we developed the local RGA approach (**Chapter 4**) as a combination of the map-based cloning strategy with the candidate gene approach. The key component of the method is BAC pool association. By analysis of genetically-defined molecular markers and a RGA

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marker on a few hundred BAC pools, one can already gain insight into the physical relationships between the genetic markers and the RGA marker. The success of the method depends on the marker saturation at the target region and the suitability of the RGA marker. Marker saturation can be achieved by a bulked segregant analysis (Michelmore *et al.*, 1991) with AFLP marker technology. The suitability of the RGA marker depended on the reliability of the clue from related species or known genotypes. The genome sequence of one Solanaceous crop can give a full picture of the genomic organization of *R* loci in the reference genome and offer such clues to clone *R* genes in related species. We believe the method has a general applicability in cloning of members of fast-evolving gene families.

Even with a completely sequenced tomato genome, it is not straightforward to use the tomato sequence to clone an *R* gene from complex *R* loci in potato. Considerably large intra-specific variation has been observed at complex *R* loci (Parniske et al., 1997; Noel et al., 1999). Let alone inter-specific variation! Besides the local RGA approach, tailor-made gene-silencing techniques might help to reveal the sequence identity of target *R* genes. Virus-induced gene silencing (VIGS) has been developed for a number of *Solanum* species (Brigneti *et al.*, 2004). VIGS with different parts of the most variable domain (LRR) will help to identify a fragment of sequence that can be used as a probe to pick up candidates from a full-length cDNA library or a binary cosmid library.

Another technical obstacle is the lack of a fast transformation system to identify the functional *R* genes from candidates. Although stable transformation is a routine procedure for potato, it takes four to six months to get the first phenotyping result. Currently Agroinfiltration or Agrobacterium transient transformation assay (ATTA) (van der Hoorn *et al.*, 2000) has not yet been efficiently applied to potato and tomato. If the *Avr* gene is known, ATTA can be applied on *Nicotiana* species by a combination of the binary *R* gene construct and the PVX *Avr* gene construct. In view of the anticipated *P. infestans* genome sequencing project (Kamoun *et al.*, 2002) and development in data-mining tools (Torto *et al.*, 2003), more *Avr* gene candidates will be discovered and benefit the cloning of corresponding *R* genes. Alternatively, by silencing the *INF1* gene in *P. infestans*, we can enable the pathogen to be more virulent on *N. benthamiana* (Kamoun *et al.*, 1998). ATTA can be performed on N. benthamiana leaves and disease resistance can be evaluated with the *INF1*-deficient isolate.

In summary, sequencing projects of potato or related species will present a full picture of *R* gene organization on the whole-genome level. This will speed up the isolation of functional *R* genes for controlling various diseases. This process

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will be further accelerated upon the development of tools in comparative and functional genomics.

Co-evolution of *R* Loci and Their Interacting Pathogens

Co-evolution is the process of reciprocal adaptive genetic change in two or more species (Fig. 1). Co-evolution can occur between any interacting populations: prey and predator, plant and herbivore, but it is expected to be particularly important in host-pathogen systems because of the intimate nature of the association and the strong selective pressures that each can exert on the other (Woolhouse *et al.*, 2002). The co-evolution between plants and pathogens in natural ecosystems is well explained with the gene-for-gene model (Flor, 1971).

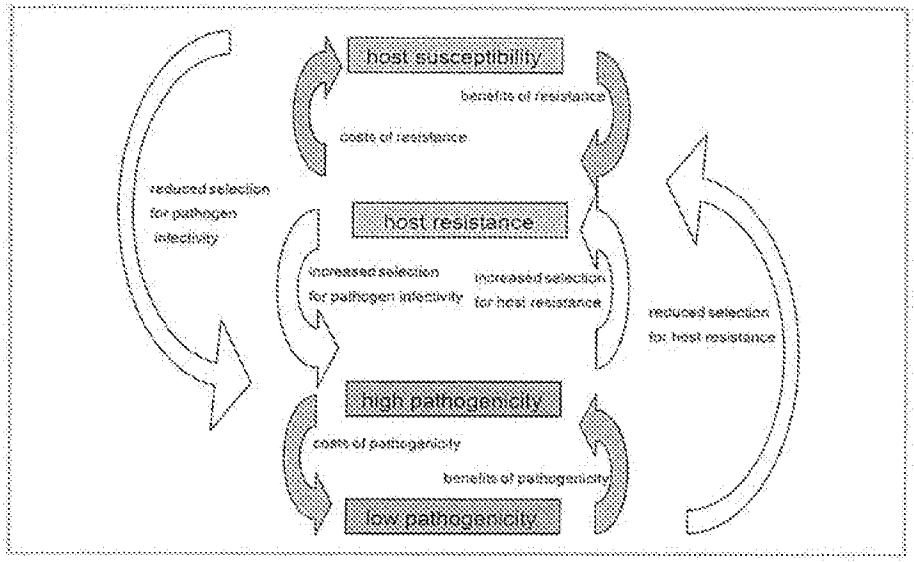


Fig.1 Schematic representation of co-evolution, emphasizing reciprocity in that changes in allele frequencies due to selection in one species impose selection resulting in changes in allele frequencies in the other. Reproduced from Woolhouse et al. 2002.

The co-evolution with pathogens must have had a direct impact on the evolution of plant *R* gene loci. Therefore, pathogens that have a great evolutionary potential should have prompted the corresponding loci to expand their *R* gene repertoire. As a contrast, plants should require fewer changes in *R* loci to cope with pathogens with less evolutionary risk. We should be able to find a correlation between the evolutionary potential of pathogens with the extent of dynamic evolution in their interacting *R* loci.

The evolutionary potential or risk of various pathogens has been investigated using parameters as mating system, gene flow, effective population

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size, and mutation rate (McDonald and Linde, 2002). Pathogens that pose the highest evolutionary potential have a mixed reproduction system, a high potential for genotype flow, large effective population sizes, and a high mutation rate. An example of this is the new *P. infestans* population with A1 and A2 mating types. The lowest risk pathogens are those with strict asexual reproduction, low gene flow potential, small effective population sizes, and low mutation rates, such as the soilborne fungus *Fusarium oxysporum* f.sp. *lycopersici* causing vascular wilt in tomato. Pathogen populations with a high evolutionary potential are more likely to overcome genetic resistance than those with a low evolutionary potential. The major *R* genes for Fusarium resistance such as *I2* have a more durable resistance than the major genes for late blight resistance such as *R3*. Interestingly, although the tomato *I2* locus is syntenic to the potato *R3* locus, we observed that the *R3* locus has undergone a more dynamic evolution than the *I2* locus as reflected in physical size, *R* gene numbers (**Chapter 4**) and functional diversity (**Chapter 5**).

Indeed, the correlation of pathogens' evolutionary risk with the speed of interacting *R* loci was observed in a few other cases. Most known *R* loci (*RPP1*, *RPP2*, *RPP5*, and *RPP8*) interacting with the oomycete *Hyaloperonospora parasitica* are complex loci with detected diversifying selection, whereas those (*RPM1*, *RPS2*, and *RPS5*) interacting with the bacterial pathogen *Pseudomonas* are single *R* gene loci under purifying selection. Another interesting case is the contrasting evolution pattern of different components in an *R* gene supercluster at the potato chromosome 5 (H.Kuang *et al*, unpublished work). Three classes of *R* genes, namely *Bs4*, *R1*, and *Prf*, were identified in the same supercluster. By comparative study of the supercluster in three genomes of *Solanum demissum*, Kuang et al. found *Bs4* and *Prf* are well conserved between different genomes and *R1* displays a more dynamic evolution in aspects of *R* gene copy numbers and sequence exchange between paralogs. This phenomenon may be explained by the pathogens with which these three clusters are interacting. *Bs4* and *Prf* confer resistance to bacterial pathogens, and *R1* is a late blight resistance gene.

This one-to-one correlation could be complicated by the fact that *R* loci could act against more than one pathogen and a given pathogen may have alternative hosts, and therefore, its evolutionary potential is not necessarily reflected in the host studied. For example, *R* genes against *P. infestans* could have distinct evolution patterns. The *S. bulbocastanum* gene *RB/Rpi-blb1* is a slow-evolving gene under purifying selection, while the *S. demissum* gene *R3a* is a fast-evolving gene under significant diversifying selection. Either *RB/Rpi-blb1* has another function in conferring resistance to another disease in *S. bulbocastanum* or *P. infestans* doesn't

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have a co-evolutionary history with the host. Interestingly we notice that *S. bulbocastanum* often grows in dry conditions, indicating its lack of co-evolution with the oomycete that needs high humidity for successful infection.

FUNCTIONAL DIVERSITY OF *R* LOCI

The *R3* locus comprises two functionally distinct *R* genes-*R3a* and *R3b*-active against the same pathogen (**Chapter 3**). Similar phenomena were discovered at the tomato *Cf2* locus that consists of almost two identical genes acting against *Cladosporium fulvum* isolates carrying *Avr2* (Dixon *et al.*, 1996), at the *Cf4* locus that contains the *Cf4* and *Hcr9-4E* genes recognizing two different *Avr* genes (Takken *et al.*, 1999), at the Arabidopsis *RPP1* locus that comprises three *R* genes with a distinct but overlapping resistance spectrum to *Hyaloperonospora parasitica* (Botella *et al.*, 1998), and at the potato *Rx* locus that harbors the *Rx1* gene for PVX extreme resistance and the *Gpa2* gene for cyst nematode resistance (van der Vossen *et al.*, 2000). Related to these, some *R* genes have dual functions. The tomato *Mi* gene confers resistance to both root-knot nematodes and aphids (Rossi *et al.*, 1998; Vos *et al.*, 1998). The Arabidopsis *RPM1* gene confers recognition to two unrelated bacterial type III effector proteins (Grant *et al.*, 1995). However, most *R* loci contain only one *R* gene of known function with or without paralogs (Hulbert *et al.*, 2001).

The evolutionary driving force of this diversity in functional contents of *R* loci remains unknown, but we can make some speculations. The guard hypothesis (Dangl and Jones, 2001) predicts the indirect recognition of Avr proteins by R proteins via guardees, the virulence targets that pathogens intend to exploit for their own benefit. It is likely that a single R protein can perceive multiple Avr proteins via the same virulence target. This gives an explanation for the dual role of some *R* genes, as demonstrated in the case of *RPM1* (Mackey *et al.*, 2002). Mathematically this explanation might be right. Most plants are resistant to most pathogens. Although preformed barriers play an important role in resistance to host or nonhost pathogens, resistance based on recognition should play a major role in the defense against diseases. The full genome sequence of Arabidopsis predicts ~150 NBS-LRR type *R* genes (Meyers *et al.*, 2003). There should be multiple times of 150 pathogen elicitors being recognized by these *R* genes. Almost all *R* genes were genetically characterized with a limited number of isolates of a limited number of pathogens and this may be the reason why their multi-facet functions are hardly unveiled. *R* genes should play an important role in nonhost resistance to keep many potential pathogens at bay.

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Most *R* loci consist of tightly linked genes with high homology. The paralogs are likely derived from the same ancestral copy by means of gene duplication and subsequent diversification. Gene duplication plays a very important role in adaptation (Hughes, 2002). After duplication, one copy can evolve more freely and gain a novel function. Or the duplicated genes subdivide the functions conferred by the ancestral gene. The functional evolution at *R* loci is driven by two major forces: diversifying selection and sequence exchange between paralogs (Michelmore and Meyers, 1998; Bergelson et al., 2001; Hulbert et al., 2001). The two copies of the *Cf2* gene may be the result of a recent duplication since their proteins only differ by three residues. The next step of divergency between duplicated genes may be as observed in the *Cf4-Hcr9-4E* pair. Then later as *Rx1* and *Gpa2* (88% a.a. identity). The *R3a* and *R3b* are from two distinct clusters of the *R3* complex, possibly the reminiscents of ancient gene duplication.

R-Gene-Free or *R*-Gene-Full, The Future of Resistance Breeding to Late Blight

Potato breeding for late blight resistance was one of the earliest practices mankind used to combat plant pathogens by means of genetic improvement (Müller and Black, 1952). Introgression of *R* genes from wild species started as early as 1909. However, the process 'reflects alternating periods of hope and despair' (Müller and Black, 1952) along with the 'boom-bust' cycles in deployment of *R* genes derived from *Solanum demissium*. Disappointed by the non-durable nature of the *S. demissum R* genes, the community either turned to other species for durable *R* genes (Hermsen and Ramanna, 1973; Colon et al., 1995; Helgeson et al., 1998; Ewing et al., 2000; Sandbrink et al., 2000), or adopted a so-called '*R*-gene-free' approach (Turkensteen, 1993; Landeo et al., 1995).

The *R*-gene-free strategy aims to explore partial resistance. To select material without detected *R* genes, one can focus on genes controlling partial resistance. This approach was embraced by CIP and some other breeders without carefully probing its scientific genuineness. First, the term '*R*-gene-free' is not scientifically correct. Even in the most susceptible cultivars such as Bintje, there could be quite many *R* genes undetected with the collections of *P. infestans* isolates. On the other hand, many so-called races 0 of *P. infestans* could be more complex than expected. For example, we found the isolate IPO-0 is not race 0 but race 3b (**Chapter 3**). If one uses IPO-0 to detect potato clones with all possible *R* genes and then throws them out from the breeding program, one may unconsciously keep the materials with *R3b*. One can never get an '*R*-gene-free' clone and totally exclude the contribution of undetected *R* genes in late blight

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resistance. Second, the approach makes a clear distinction between major *R* genes with clear resistance phenotypes and minor *R* genes with partial resistance and correlates this distinction with durability. This correlation is superficial. In many other pathosystems, major *R* genes can be durable, such as the pepper *Bs2* gene for *Xanthomonas* resistance (Kearney and Staskawicz, 1990), and the tomato $Tm2^2$ gene for TMV resistance (Lanfermeijer *et al.*, 2003). Although the molecular basis of partial resistance is poorly understood, some paralogs of major *R* genes act as quantitative resistance loci (QRLs) in tomato and rice (Parniske et al., 1997; Wang et al., 1998; Takken et al., 1999). In potato, QRLs often cluster with major *R* genes (Gebhardt and Valkonen, 2001), suggesting their similar identity and mode of action. In addition, partial resistance in some old potato cvs. such as Pimpernel also display race-specificity to some extent (Flier *et al.*, 2003). Third, this approach totally ignores the contribution of major *R* genes to the overall resistance of potato cultivars. *R* genes are often present in most resistant progeny in a resistant x susceptible cross (Stewart *et al.*, 2003). About 80% of cvs. of the former Federal Republic of Germany have *S. demissum* pedigrees due to the *R* genes derived from the wild species (Ross, 1986). A recent linkage disequilibrium study in 600 potato cvs. demonstrated that the presence of the *R1* gene is positively correlated with higher field resistance (Gebhardt *et al.*, 2004). Last, this approach is also practically unsuccessful since partial resistance is often unfavorably linked to late maturity and low vigor (Collins et al., 1999; Simko, 2002).

How about the *R*-gene-full strategy? Despite disappointment by the non-durable nature of known *R* genes, some breeders never stopped their attempt to accumulate *R* genes into cultivars with the expectation that pyramiding of several *R* genes into the same cultivars would enable more durable resistance. The process of pyramiding involves tremendous effort but only resulted in the release of few cultivars such as the Dutch cv. Escort and the Scottish cv. Stirling with reasonably good resistance. Yet these cultivars are not grown in a considerably large acreage. *R* genes may have costs, as demonstrated in the case of the Arabidopsis bacterial resistance gene *RPM1* (Tian *et al.*, 2003). Therefore, if a potato cultivar has too many *R* genes, it likely gets a fitness penalty such as lower vigor. Most crop species might have lost their *R* genes in the process of domestication. Resistance might be negatively selected in favor of high yield and better flavor. A balance between resistance and other agronomic traits has to be maintained.

R gene polyculture or multiline is an alternative solution to the vulnerability of monocultured crops to diseases (Wolfe, 1985; Jones, 2001; Mundt, 2002). Cultivar mixtures have proven to be a significant measure in the control of rice blast (Zhu et al., 2000). Some experiments indicated that cultivar mixtures also

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works in the potato-*P. infestans* pathosystem (Garrett and Mundt, 2000). However, *R* gene polyculture in a real sense has never been practiced in potato as it is difficult to create a polyculture in potato due to its teirasomic inheritance and inbreeding depression. The GMO approach is so far the only possible effective method to create a mixture of potato cultivars that only differ by the *R* genes. Since several late blight *R* genes have been cloned and particularly since we discovered that *R3* has multiple allelic versions to allow allele-mining (**Chapter 5**), we should expect that a dozen *R* genes will be available in the near future. It is a good time now to put *R* gene polyculture for late blight control to a serious experimental test.

The ecological implication associated with the *R* gene polyculture concept is to make the pathogen population heterozygous by equipping hosts with heterozygous resistance specificities. This is to create equilibrium between the host and the pathogen, by allowing part of the host being exploited by the pathogen and keeping the remaining healthy. To maintain the equilibrium at an economical threshold, we should soberly realize that resistance conferred by *R* genes is not enough. Instead, we need to adopt integrated disease management that combines the smart deployment of host resistance, cultural practices, and a limited application of pesticides.

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Summary

Potato is the most important non-cereal crop in the world. Late blight, caused by the oomycete pathogen *Phytophthora infestans*, is the most devastating disease of potato. In the mid-19th century, *P. infestans* attacked the European potato fields and this resulted in a widespread famine in Ireland. Late blight remains the No.1 constraint to potato production and causes a yearly multi-billion US$ loss globally. In Europe and North America, late blight control heavily relies on the use of chemicals, which is hardly affordable to farmers in developing countries and also raises considerable environmental concerns in the developed countries.

Use of host resistance is ecologically the most sustainable way of disease management. Disease resistance (*R*) genes exist in a wide range of wild species of the genera *Solanum*. One wild species, *Solanum demissum*, became the donor of most characterized *R* genes due to its crossability with the cultivated species *S. tuberosum*. However, these race-specific genes (*R1-R11*) didn't enable satisfactory protection under the current deployment scheme. Breeders either turned to other wild species or adopted a so-called '*R*-gene-free' approach to explore quantitative resistance. Unfortunately, neither alternative offered a solution to late blight control and did not result in commercial release of cultivars with durable resistance.

At the end of the 20th century, biological research entered the genomics era, landmarked by the Human Genome Project and by the Arabidopsis and rice genome sequencing initiatives. Genomics also became the new frontier of research in potato and *P. infestans*. This scientific development is deepening and broadening our understanding of the biology of the host and the pathogen and is facilitating isolation of key genes involved in the interaction. The genetically modified organism (GMO) strategy allows a much more efficient application of these genes than time-consuming conventional breeding. This thesis deals with the isolation, characterization, and deployment of host *R* genes with the expectation to achieve an ecologically and economically sound control of late blight.

The potato-*P. infestans* interaction follows the gene-for-gene model, that is, resistance only occurs when a host *R* gene and its corresponding avirulence (*Avr*) gene in the pathogen are both present. Several disease testing methods have been developed for determination of the gene-for-gene interaction between potato and

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P. infestans. *In vitro* inoculation was developed as a quick, space-effective, and accurate assay (**Chapter 2**). The method exploits the amenability of potato for tissue culture and the suitability of the *in vitro* environment for late blight disease development. Its specificity and reliability was confirmed by comparison with the well-established detached-leaf assay. Currently, in vitro inoculation is routinely used in phenotyping of segregating populations, resistance testing of transformants for functional complementation, and screening of new *R* genes in a wide range of wild germplasms.

The investigation of host resistance was focused on the *R3* complex locus on the distal part of chromosome 11. The *R3* complex locus segregated in a potato mapping population, which was used to construct the potato ultra-high dense (UHD) map saturated with over 10,000 amplified fragment length polymorphism (AFLP) markers. Using a population of 1748 plants, we constructed a high-resolution genetic map at the *R3* complex locus. The combination of fine-mapping and accurate disease testing with specific *P. infestans* isolates resulted in the unexpected discovery that the *R3* complex locus is composed of two functionally distinct genes, *R3a* and *R3b*, which are 0.4 cM apart and have both been introgressed from *S. demissum* (Chapter 3). Each gene was localized into a genetic interval of 0.25 cM, providing the starting point for map-based cloning.

Plant *R* gene families undergo fast evolution, resulting in considerable intra- and inter-specific variation. Plant disease resistance (*R*) loci frequently lack synteny between related species of cereals and crucifers but appear to be positionally well conserved in the Solanaceae. Comparative genomics provides a tool to utilize the exponentially increasing sequence information from model plants to clone agronomically important genes from less studied crop species. We were keen to investigate whether this tool can enable new *R* gene cloning by a case study. The comparative study revealed that the potato late blight *R3* locus and the tomato *Fusarium* wilt *I2* locus were derived from an ancestral locus involved in plant innate immunity. We adopted a local RGA approach using DNA sequences of the *I2* gene to isolate the *R3a* gene (Chapter 4). *I2* and *R3a* share 88% and 83% identities at the DNA and protein level, respectively. *R3a* is a member of the *R3* complex locus. Comparative physical mapping disclosed that the potato *R3* complex locus underwent a significant expansion after divergence from tomato without disruption of the flanking colinearity. This expansion resulted in an increase in the number of *R* genes and functional diversification. Interestingly, the differential evolution of the ancient *R* locus in the two closely related species is well correlated with the contrasting evolutionary potentials of the pathogens with which *I2* and *R3* interact.

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Fusarium oxysporum is a soilborne fungus with low genotype diversity, whereas the late blight pathogen *P. infestans* is notorious in its ability to move and mutate.

An intriguing question is why *S. demissum*, a species that appears to contain only easily-broken *R* genes, can display durable resistance at the population level in its natural environment. The polymorphism of parasite recognition capacity in a host population will restrict most isolates of the parasite to grow on most hosts. Allelism is an efficient way of creating recognition polymorphism. We made another unexpected discovery that the *R3* complex locus has very high allelic diversity and that the *R5~R11* resistant specificities all contain a distinct allelic version (Chapter 5). Sequence exchange between alleles and diversifying selection are the major driving forces of this allelic recogntion polymorphism to *P. infestans*. Remarkably, the genomic structure of the *R3* complex locus favors the creation of new resistance specificity by reshuffling of elements from the two clusters (*R3a* and *R3b*). The multiple allelism of the *R3* complex locus may be a natural mechanism of *S. demissum* to suppress late blight epidemics and should be mimicked in resistance breeding. We suggest that *R*-gene polyculture via the GMO approach should be the future paradigm of *R* gene deployment in late blight control.

Potato breeding for late blight resistance was one of the earliest mankind practices in combating plant pathogens by means of genetic improvement but the disease has not been controlled by resistance breeding so far. The non-durable nature of *S. demissum R* genes apparently disappointed most breeders and the blame of easily-broken *R* genes even led to the unsuccessful *R*-gene-free approach. However, from an evolutionary and ecological point of view, single *R* genes can never defeat pathogens such as *P. infestans* with extremely high evolutionary risk. We propose that the community rethinks its strategy of *R* gene deployment in late blight disease management. *R* gene monoculture is obviously not recommended. *R* gene pyramiding is currently practiced by breeders but this strategy is basically the same as *R* gene monoculture since it creates uniformity in host resistance specificity in the field, which will be eventually broken by the fast-evolving pathogen. *R* gene polyculture should be the strategy of the future. However, we should soberly realize that host resistance might be high enough for survival of the plant population but it alone might never offer a protection that meets the economical threshold. Integrated pest management should include *R* gene polyculture as the central element, cultivation measures, and limited chemical applications.

To provide this central element, we need to clone more *R* genes. Our discovery of the multiple allelism of the *R3* potato late blight resistance complex and molecular characterization of one of its allelic versions will offer a possibility to

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clone a dozen *R* genes in the near future and then to deploy them in a potato field using marker-free GMO techniques.

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Samenvatting

Wereldwijd is aardappel het belangrijkste voedingsgewas, naast de granen. De aardappelziekte, veroorzaakt door de oomyceet *Phytophthora infestans*, is de meest verwoestende ziekte in dit gewas. Halverwege de 19e eeuw tastte *P. infestans* de Europese aardappelvelden aan waardoor in Ierland een dramatische hongersnood ontstond. De aardappelziekte *Phytophthora* blijft voor de aardappelproductie probleem nummer 1 en veroorzaakt wereldwijd jaarlijks vele miljarden Euro's verlies. De beheersing van *Phytophthora* berust in Europa en N. Amerika op het gebruik van milieubelastende chemicaliën, maar boeren in ontwikkelingslanden kunnen zich deze nauwelijks veroorloven.

Het gebruik van resistente rassen is ecologisch gezien de meest duurzame manier van ziektebeheersing. Resistentiegenen komen voor in een groot aantal wilde soorten van het genus *Solanum*. *Solanum demissum*, is door zijn kruisbaarheid met de cultuuraardappel *S. tuberosum*, de donor van de best gekarakteriseerde *R* genen geworden. Maar de manier waarmee deze genen (*R1-R11*) tot nu toe ingezet zijn in de teelt, heeft slechts kortdurende bescherming opgeleverd. Veredelaars hebben ook sporadisch andere wilde soorten gebruikt, of hebben zich gericht op partiële resistentie in een zgn. "*R* gen vrije" methode. Ongelukkigerwijs hebben geen van beide alternatieven een oplossing voor de beheersing van de aardappelziekte gebracht, en commerciële rassen met een duurzame resistentie zijn nog steeds niet voor handen.

Aan het eind van de 20e eeuw is het biologisch onderzoek in het "genomics tijdperk" terecht gekomen. Dit mondde uit in o.a. het Humane Genoom project en in genoom sequencing projecten van *Arabidopsis* en rijst. Ook bij aardappel en *P. infestans* heeft genomics onderzoek zijn intrede gedaan. Deze wetenschappelijke ontwikkeling verdiept en verbreedt ons begrip van de biologie van de gastheer en het pathogeen, en maakt moleculaire isolatie mogelijk van sleutelgenen die bij deze interactie betrokken zijn. De GMO (genetisch gemodificeerde organismen) technologie staat een veel efficiënter gebruik van deze genen toe dan de tijdverslindende conventionele veredeling. Dit proefschrift gaat over de isolatie, karakterisering en toepassing van resistentiegenen (*R* genen) in de verwachting hiermee tot een meer ecologisch en economisch aanvaardbare beheersing van de aardappelziekte te komen.

De interactie tussen aardappel en *P. infestans* volgt het gen-om-gen model. Dit betekent dat de plant alleen resistent is als zowel het *R* gen in de plant en het

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corresponderende avirulentiegen (*Avr* gen) in het pathogeen aanwezig zijn. Verschillende ziektetoetsen worden gebruikt om de gen-om-gen interactie tussen aardappel en *P. infestans* te kunnen vaststellen, maar deze zijn vaak tijdrovend. In **Hoofdstuk 2** wordt de ontwikkeling van *in vitro* inoculatie beschreven als een snelle, ruimte besparende en betrouwbare methode. Dit systeem is gebaseerd op de geschiktheid van *P. infestans* om onder *in vitro* omstandigheden te groeien, en maakt gebruik van het feit dat aardappel zeer goed *in vitro* vermeerderd kan worden. De specificiteit en betrouwbaarheid van deze methode kon worden vastgesteld door deze te vergelijken met de algemeen bekende toets met afgesneden bladeren. Op dit moment wordt *in vitro* inoculatie in het laboratorium op routine basis toegepast bij zowel het fenotyperen van splitsende populaties, het toetsen van resistentie bij transformanten voor functionele complementatie, evenals het screenen van wilde *Solanum* soorten voor aanwezigheid van nieuwe *R* genen.

Het onderzoek naar resistentie richt zich op het *R3* locus, dat op het distale deel van chromosoom 11 gelegen is. Dit locus splitst uit in een populatie die gebruikt is voor het genereren van een ultra dichte genetische kaart, die verzadigd is met meer dan 10.000 AFLP (amplified fragment length polymorphism) merkers. Vervolgens is een populatie van 1748 planten gebruikt om een hoge resolutie genetische kaart van het *R3* locus te maken. De combinatie van fijn-kartering en betrouwbare resistentietoetsen met specifieke isolaten van *P. infestans* resulteerde in de onverwachte ontdekking dat het *R3* locus bestaat uit twee functioneel verschillende genen, *R3a* en *R3b*. Deze genen liggen 0.4 cM van elkaar en beide zijn afkomstig van *S. demissum* (**Hoofdstuk 3**). Beide genen zijn gelokaliseerd binnen genetische intervallen van 0.25 cM, die het startpunt voor de "map-based" klonering vormden.

R genfamilies van planten ondergaan vaak een snelle evolutie, die uiteindelijk resulteert in een aanzienlijke intra- en interspecifieke variatie. Resistentieloci van granen en Cruciferen missen vaak positionele overeenkomst ('synteny') tussen verwante soorten, maar bij de Solanaceae lijken deze goed geconserveerd. Vergelijkende genomica geeft steeds meer de mogelijkheid in handen om de exponentieel toenemende sequentie-informatie van modelplanten te gebruiken voor het kloneren van landbouwkundig belangrijke genen bij minder intensief bestudeerde cultuurplanten. Wij wilden weten of deze mogelijkheid gebruikt kon worden om een nieuw *R* gen te kloneren. Het vergelijkende onderzoek liet zien dat het aardappel *R3* gen tegen *P. infestans* en het *I2* gen van tomaat tegen *Fusarium oxysporum* afkomstig waren van één oorspronkelijk gen dat in de plant immuniteit veroorzaakt. Voor de klonering van het *R3a* gen hebben wij een plaatsgerichte RGA (Resistance Gene Analogue) benaderingswijze toegepast door DNA-specifieke

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sequenties van het *I2* gen te gebruiken (**Hoofdstuk 4**). *I2* en *R3a* hebben 88% en 83% homologie op respectievelijk DNA- en eiwitniveau. *R3a* is een lid van het complexe *R3* locus. De fysische kaart gaf aan dat het *R3* locus van aardappel een aanzienlijke expansie heeft ondergaan t.o.v. het locus op tomaat, waarbij de flankerende co-lineariteit intact is gebleven. Door deze expansie is er een toename van het aantal *R* genen én van functionele diversiteit. De differentiële evolutie van het oorspronkelijke *R* locus in tomaat en aardappel is goed gecorreleerd met de contrasterende evolutionaire potentie van de betrokken pathogenen waarmee *I2* en *R3* interactie aangaan. *F. oxysporum* is een grondgebonden schimmel met een relatief lage genetische diversiteit, terwijl *P. infestans* zeer berucht is om haar vermogen zich te verplaatsen en om te muteren.

Een intrigerende vraag is waarom de soort *S. demissum*, die schijnbaar alleen eenvoudig te doorbreken *R* genen bevat, in haar natuurlijke omgeving duurzaam resistent is. De polymorfie op het niveau van pathogeenherkenning in een plantenpopulatie zal de meeste isolaten van het pathogeen verhinderen te groeien op de meeste planten. Een efficiënte manier om polymorfie op het punt van herkenning te verkrijgen is allelie. Een onverwachte ontdekking in ons onderzoek is, dat het *R3* locus een hoge allelische diversiteit bezit en dat de *R5-R11* resistenties allemaal een andere allele versie van het *R3* locus bevatten (**Hoofdstuk 5**). Sequentie-uitwisseling tussen allelen en diversificerende selectie zijn de belangrijkste drijvende krachten achter deze herkenningspolymorfie tegen *P. infestans* op allelniveau. Opvallend is dat de genoomstructuur van het *R3* locus het ontstaan van nieuwe specifieke resistenties bevordert door menging ('reshuffeling') van elementen van de twee clusters (*R3a* en *R3b*). De multi-allelie van het *R3* locus zou een natuurlijk mechanisme van *S. demissum* kunnen zijn om *P. infestans* epidemieën te onderdrukken. Deze benaderingswijze is in de resistentieveredeling tot nu toe nog niet gebruikt.

Resistentieveredeling in aardappel tegen *Phytophthora* was een van de eerste toepassingen van de mens om met behulp van genetische methoden de strijd tegen pathogenen aan te binden, maar tot op de dag van vandaag is deze ziekte zonder chemische middelen niet onder controle. De niet-duurzame aard van de *R* genen uit *S. demissum* heeft veel veredelaars teleurgesteld en was er zelfs de oorzaak van om met de (eveneens onsuccesvolle) "*R* gen vrije" benadering te beginnen. Echter, vanuit evolutionair en ecologisch oogpunt gezien kunnen enkelvoudige *R* genen pathogenen die een hoog evolutionair risico in zich dragen, zoals *P. infestans*, nooit beheersen. Op grond van dit proefschrift moet het gebruik van *R* genen bij het beheersen van *Phytophthora* heroverwogen worden. Het gebruik van enkelvoudige *R* genen wordt afgeraden. *R* gen stapeling is op dit moment de meest gevolgde

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benaderingswijze, maar deze is in principe vergelijkbaar met het toepassen van enkelvoudige genen, omdat het met uniformiteit van de resistentie in het gewas gepaard gaat die door het snel veranderende pathogeen eventueel doorbroken kan worden. Het gebruik van meerdere *R* genen in gemengde rassen, gebaseerd op één basisras, zou de toekomstige strategie moeten zijn. Hierbij is toepassing van GMO om gekloneerde genen binnen te brengen onontbeerlijk. Het is te verwachten dat alleen deze benaderingswijze niet altijd de mate van bescherming geeft die in een moderne economische teelt noodzakelijk is. Dit betekent dat het een onderdeel moet zijn van een geïntegreerde methode, waarin het toepassen van *R* genen een centraal element is naast het beperkte gebruik van chemicaliën.

Er zijn veel meer gekloneerde *R* genen nodig om tot de beschreven benaderingswijze te komen. De ontdekking van het complexe *R3* locus voor *Phytophthora*-resistentie en de moleculaire karakterisering van één van de allele versies geeft de mogelijkheid om op korte termijn een groot aantal andere *R* genen te kloneren, en deze vervolgens met behulp van de merker-vrije GMO benaderingswijze toe te passen in het veld.

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博士论文

英文题目

Discovery and characterization of the major late blight resistance complex in potato

genomic structure, functional diversity, and implications

中文题目

马铃薯抗晚疫病主效基因位点的发现和分析

基因组结构、功能多态性及对抗病育种的影响

黄三文

导师：Evert Jacobsen 教授，Richard G.F. Visser 教授
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马铃薯是世界上最重要的非禾本科作物。晚疫病是由卵菌 *Phytophthora infestans* 引起的，是马铃薯最严重的病害。十九世纪中叶该病害曾在欧洲马铃薯生产区广泛流行，导致了举世闻名的爱尔兰大饥荒（The Great Irish Famine），至今仍然是马铃薯生产的第一限制因素，在全球造成数十亿美元的损失。在欧洲和北美，晚疫病防治严重依赖于化学杀菌剂，已经形成了严重的环境问题。发展中国家的农民则无力购买这些昂贵的杀菌剂。

利用寄主抗性是生态学上最安全最可持续的病害防治策略。抗病基因普遍存在于马铃薯的近缘野生种中。其中一个野生种 *Solanum demissum* 由于可以和栽培种 *S. tuberosum* 杂交而成为目前已知抗病基因的主要来源。但是，这些主效抗病基因（*R1-R11*）在目前的田间施用策略下并没有提供足够的防治功能。育种家们或者到别的野生种中寻找具持久抗病的主效基因，或者采用所谓的‘非主效抗病基因（*R*-gene-free）’策略利用微效数量性抗病基因。不幸的是，这两个替代措施都没有能够成为晚疫病防治的有效手段，也没有育成具持久抗性的品种。

二十世纪后期，人类基因组计划和拟南芥和水稻基因组计划标志着生物科学研究进入了基因组学时代。基因组学也迅速成为马铃薯和晚疫病研究的前沿，深化和扩展了我们对寄主（马铃薯）和病原（晚疫病菌）生物学的认识，促进了控制寄主-病原互作的关键基因的克隆。和常规育种相比，转基因技术能够使这些关键基因很快地用于晚疫病防治。本论文内容涉及到抗晚疫病基因的克隆、分析和田间施用策略，以期实现一套生态安全且经济可行的晚疫病防治新策略。

马铃薯-晚疫病菌的互作符合著名的‘基因对基因’假说。该假说提出抗病反应是寄主和病原相互识别的结果，只有寄主的抗病基因和病原中相对应的非毒力基因同时存在并表达时，寄主才会有抗病反应。前人已经发明了几种研究马铃薯-晚疫病菌‘基因对基因’关系的接种方法，但它们都不适合于高效大规模的抗病鉴定。我们所发明的无菌苗接种法（*In vitro* inoculation）是一种快速、高效和准确的抗病鉴定方法（第二章）。该方法利用了马铃薯容易组培的特点和无菌培养条件很适合于晚疫病发病这两个有利条件。其特异性和可靠性在与公认的离体叶片接种法（Detached-leaf assay）比较中得到证明。目前，无菌苗接种法已经成为分离群体抗病鉴定、转基因苗功能互补鉴定和筛选抗病野生种质资源的常规方法。

本论文所研究的抗病基因位点是 *R3*。该遗传位点已被定位在马铃薯第 11 号染色体的末端，并被发现分离于用以创建马铃薯超高密图谱（Ultra-High Density map）的群体里。该图谱由瓦赫宁根大学育种系主持完成，共标定了 10,000 扩增片段长度多态性（amplified

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fragment length polymorphism, AFLP）标记。在 UHD 图谱中，我们发现了 43 个和 *R3* 共分离的 AFLP 标记。通过一个大分离群体（1748 株），我们创建了 *R3* 的高分辨率遗传图谱。结合此高分辨率图谱和利用特异性的晚疫病菌株鉴定，我们发现了一个预想不到的现象：*R3* 并非只有一个抗病基因，它是由两个抗谱独特的抗病基因组成的（第三章）。这两个基因，*R3a* 和 *R3b*，相距 0.4 cM，都来源于野生种 *S. demissum*。*R3a* 和 *R3b* 被分别定位于 0.25 cM 的遗传区间里，为通过图位克隆法分离它们提供了很好的基础。

抗病基因家族经历了非常快的进化，导致了非常大的种内和种间变异。在禾本科和十字花科中，植物抗病基因位点（*R* 位点）在相关物种的进化同源性（Synteny）往往由于快速进化而丢失。相反，*R* 位点在茄科的相关物种中却存在相当的保守性。目前，从模式植物中产生的 DNA 和蛋白质序列信息呈指数形式增长。比较基因组学为利用这些信息来克隆非模式作物的重要农业性状基因提供了工具。但是至今利用进化同源性克隆抗病基因尚是比较基因组学的难点之一，尚无先例。我们现提供首例利用比较基因组学工具克隆一个新的功能性的抗病基因（第四章）。比较遗传作图揭示了马铃薯抗晚疫病基因位点 *R3* 和番茄抗根腐病基因位点 *I2* 来源于同一个控制植物内生免疫系统的原始位点。我们采用了一个新方法-抗病基因局部同源法（Local resistance gene analog），利用已知的 *I2* 基因序列克隆了 *R3* 位点的其中一个基因 *R3a*。在 DNA 和蛋白质水平上，*I2* 和 *R3a* 分别有 88%和 83%的同一性。比较物理作图揭示在和番茄分支以后，马铃薯的 *R3* 位点经历了显著的基因组扩增，但是此扩增并没有影响到 *R3* 两侧基因组区域和番茄的等线性（Colinearity）关系。此扩增导致了抗病基因数目的增加和功能的多样化。更有意义的是，*I2-R3* 这个原始位点在番茄和马铃薯中的不同的进化命运是和与其互作的病原微生物的进化潜力紧密相关。和 *I2* 位点互作的病原菌 *Fusarium oxysporum* 是一种土传真菌，移动距离有限，病菌群体基因型变异小，进化潜力小；而和 *R3* 位点互作的晚疫病菌可以通过空气传播，病菌群体基因型变异非常大，进化潜力极大。

一个值得深思的问题是：从 *S. demissum* 导入到栽培种马铃薯的抗病基因（*R1-11*）都很容易被晚疫病菌克服，但是这个野生种在自然环境下却有持久的抗病性。群体遗传学研究表明抗病基因多态性将限制病原的大部分菌株在寄主群体里的流行。我们发现了另外一个预想不到的现象：抗晚疫病位点 *R3* 有非常多的等位基因，并且 *R5-R11* 都是 *R3* 的等位基因（第五章）。等位基因之间的序列交换和达尔文式正向选择（Darwinian positive selection）是 *R3* 位点等位基因多态性的主要进化驱动力。引人注目的是，*S. demissum* 可以通过 *R3* 位点的两个部分-*R3a* 和 *R3b* 的重组来产生新的抗病特性。*R3* 位点的多重等位基因很可能是 *S. demissum* 控制晚疫病流行的天然机制。此机制应该在抗病

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育种中被模仿。我们建议近等抗病基因混合系（*R*-gene polyculture），即一个除抗病基因不同外其它农艺性状均相同的混合群体，应该成为晚疫病防治中抗病基因田间施用的策略。在马铃薯育种中，回交育种将导致严重自交衰退，因此创造近等抗病基因混合系的唯一手段是克隆野生种的抗病基因并通过转基因技术将其导入到优良栽培品种中。

马铃薯抗晚疫病育种是人类历史上最早利用遗传改良来控制植物病害的实践之一，但至今尚未取得成功。*S. demissum* 中抗病基因的非持久性显然使大多数育种家们失望。这种失望甚至导致了部分育种家采用了不成功的‘非主效抗病基因’策略。然而从进化学和生态学的角度来看，对于晚疫病菌这种进化潜力巨大的病原，单个抗病基因永远不可能产生持久抗病性。因此我们不能埋怨抗病基因不持久，相反我们建议马铃薯育种界重新思考抗病基因的田间施用策略。抗病基因单系(*R*-gene monoculture)显然已被历史证明是无效的。抗病基因累加法(*R*-gene pyramiding)，即在同一个品种中导入多个抗病基因，是目前育种界采用的策略，但是此策略在本质上和抗病基因单系没有区别，因为它在田间形成了单一的抗病特异性，将迟早会被晚疫病菌这一进化能力极强的病原克服。近等抗病基因混合系是将来应该采取的策略。但是，我们应该清醒地意识到寄主抗病性的水平也许能够高到足以使植物群体繁衍下去，但仅依赖寄主抗病性本身不能使植物保护达到经济学的阈值。晚疫病综合病害防治（Integrated pest management）应该将近等抗病基因混合系作为中心，辅之以栽培防病措施（如轮作）和化学杀菌剂的有限使用。

为了育成近等抗病基因混合系这个中心元素，我们需要克隆更多的抗病基因。我们关于抗晚疫病 *R3* 位点有多个等位基因的发现（第五章），以及我们对其中一个等位基因的克隆和详细分析（第三、四章），将使得克隆近十个抗晚疫病基因成为可能。在不久的将来，我们将应用无标记基因（Marker-free，即没有抗生素标记基因）的转基因技术育成近等抗病基因多系在田间施用。

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Acknowledgements

The autobiography of Konosuke Matsushita, the founder of Matsushita Electric Corporation, influenced me a lot. I read it in 1996, just after I came out of school and started my career as pepper breeder in the Institute of Vegetables and Flowers, Chinese Academy of Agricultural Sciences (IVF-CAAS). From this book, I picked up the value of being thankful among many others. The past four years is a wonderful chapter in my life journey, where I met so many amazing people whom I should never forget to thank.

My first thank goes to Prof. Evert Jacobsen, Prof. Qu Dongyu, and Prof. Zhu Dewei. Thanking to their effort, the long-term link between CAAS and WUR has been maintained, which made my study in Wageningen possible and will be intensified upon my return.

The story of Evert and me resembled the one of a dominant father and a rebellious son, in which the initial bitter fight turned out as reciprocal respect and appreciation in the end. Evert taught me the art of 'positive fighting'. He is my personal promoter: whenever he could, he will promote (sometimes boast) me in any circumstance! I will have to prove that his promotion on me is not too far from the fact.

When I reported my latest results to Richard Visser, his eyes were always widely open. "Exciting!" "Go ahead!" "Why not!" Richard backed up almost all my scientific ideas, although many of them turned out to be wishful thinking. His trust and solid support are essential to my PhD work.

If I am a speeding car, Vivian is my brake! I often came up with 10 ideas in one hour, and she often told me that 9.9 of them were indeed not that smart, and after the screening we finally had some nice ideas together. She virtually taught me everything about scientific writing. In the second half my PhD, Edwin offered me intensive supervision. We used to have a meeting in Friday morning, which I enjoyed a lot. Without Edwin, the cloning of the *R3a* gene would be a mission of impossible. He guided me through all the ins and outs of molecular cloning.

Herman's group is full of characters. I am one of them, and therefore, Herman knows that Chinese are not identical. Herman's criticism about my scientific works is what I appreciated a lot. I believe in Science criticism is a parameter of friendship.

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I benefited from the potato pedigree database created by Ronald. Dirk Jan was a key man, who handles almost all plant material for my experiments.

I like very much the atmosphere of work discussion in the resistance group every Tuesday morning. I wish my future group could have the vivid discussion too. Pim taught me that the quality of presentation is largely a matter of attitude.

I thank all the technicians in the laboratory to keep the lab smoothly running. Fien is a professional a manager. In the beginning I was not used to her style but later on I appreciated it completely. Isolde kindly helped me with genetic transformation. Dirk's help on resistance testing on various populations was big relief to me in my last few months in the laboratory.

I have a pleasant stay in Plant Breeding, thanking to many other colleagues and especially to my roommate Taeho and Maarten. If I forget to mention you, please forgive me! I wrote this 1 hour before I sent it to print!

There are more on my list! Francine Govers spent a lot of energy to make critical comments on my publications and thesis. Rob Weide, thank you for not getting annoyed from my endless request for *Phytophthora* isolates! I would not have been interested in plant pathology if I had not read one of Prof. Pierre de Wit's publications. He gave a close watch my research in Wageningen and endorsed me for my application to the faculty position in CAAS.

The communication with Sophien Kamoun (Ohio State University) was always pleasant. His efficiency was really incredible! I sent him my article in the morning and he replied me in the afternoon with full edition and many good ideas. My cooperation with Barbara Baker and Kuang Hanhui (both in UC Berkeley) was enjoyful. I thank Hanhui for teaching me the knowledge of evolution.

My students are all the essential contributors of this thesis: Jeroen for the *R3* genetic mapping, Ningwen for the *R3* physical mapping, Dinah for the *R3a* transformation, and Tao for the *R3* multiple allelism. I am grateful to them! I am happy that Jeroen and Ningwen became PhD students too, that Dinah is doing fine with her MSc in Wageninen, and that Tao found a job in the Chinese Division of Ing Bank.

My chairship of Chinese Association of Students and Scholars in Wageningen (CASSW) from Dec. 2002 to April 2004 was a real flavor to my busy PhD life in Wageningen. I steadily fought for the honor and interest of the Chinese community and I won back appreciation. My team members (Qin Ling, Zhu Xueqin, Shi

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Songkai, Li Tao, Meng Xiangyan, Bai Zhanguo, Lin Ke, Zhang Ningwen, An Ran, Lei Ying, Ren Xinying etc) are great people. We achieved so much together. Liu Chun-Ming and Huang Jiang and their family were also my enthusiastic supporters. I am especially grateful to Prof. Tong Guangwu and Prof. Dong Huiqing from the Chinese Embassy, who offered unlimited support to my turn in CASSW. Within the WUR, I have speical thanks to rector magnificus Prof. Speelman and Mr. Rien Bor for their kind help to CASSW. I thank Yvonne de Hilster from Wb offered CASSW quite some publicity.

One of the treasures that my wife and I have in Wageningen is our friends. The family of Wang Ying and Liu Chun-Ming, He Qian and Huang Jiang offered us timely help after the birth my daughter Yike. Yike was also loved by her Oma Mrs. Ancor Jacobsen and Mrs. Carol Lynn Crow.

I didn't grow up in a rich family, but I was never short of love. The love from grandma, parents, uncles and aunts, brothers and cousins was one the largest attractions for me to return to China.

Although there are many regrets and failures in my thesis, I should be a lucky PhD student. I found that the *R3* locus segregates in the mapping population for the ultra high dense map that the locus consists of two genes and that *R5-11* are allelic to *R3*. All these key findings of my thesis are results of experiments without too much effort. However, these lucks added together can not compare with a tiny part of the one I had more than 12 years ago. It was a summer day and I was in my uncle's house and planned to go back to my parents. My aunt insisted that I had dinner with them before going home. If I didn't follow her advice, I would not have had the chance to meet the amazing Wang Min, who became my girlfriend for 4 years, my wife for 8 years, mother of my litter angel Yike for >1 year, and my lover 4ever.





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Curriculum Vitae

Sanwen Huang was born on September 10, 1971 in Hunan, China. In Beijing Agricultural University, he obtained his BSc as Horticulture major in 1993 and MSc as Plant Sceince major in 1996. From 1996 to 2000, he worked as a pepper breeder at Institute of Vegetables and Flowers, Chinese Academy of Agricultural Sciences (IVF-CAAS).

From September 2000 to Jan 2005, he works as a PhD student in the Laboratory of Plant Breeding, Graduate School of Experimental Plant Sciences (EPS) of Wageningen University and Research Centre. The thesis is the results of four years of research carried out in this laboratory, which was financed by fellowship from the chair of Plant Breeding of Wageningen University.

After his defense for the doctorate, he will return to IVF-CAAS and set up a lab on Molecular Genetics of Solanaceous Crops.

Publications

Huang, S., van der Vossen, E., Kuang, H., Vleeshouwers, V.G., Zhang, N., Borm, T.J., Van Eck, H.J., Baker, B., Jacobsen, E., and Visser, R. Comparative genomics enabled the cloning of the *R3a* late blight resistance gene in potato. Accepted by the Plant Journal

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Training and supervision plan of the Graduate School Experimental Plant Sciences

1. Participation in postgraduate courses and workshops
   a. EPS Autumn School "Interaction between plants and attacking organism".
   b. Scientific writing.
   c. Skills in Scientific presentation.
   d. Endnote 7

2. Participation in international meetings:
   a) Symposium Durable Disease Resistance, Ede, the Netherlands (2000), attendence.
   b) Global Initiative of Late Blight & European Association Potato Research, Hamburg, Germany (2002), poster presentation.
   c) Plant and Animal Genomes XII, San Diego, California, USA (2004), poster presentation.
   d) NSF Potato genomics annual meeting, San Diego, California, USA (2004), oral presentation.

3. Participation in national meetings
   a) EPS flying and other seminar (2000-2004).
   b) Annual EPS theme symposia (2000-2004), poster and oral presentation.
   c) ALW (Earth and Life Sciences) meetings (2004), poster and oral presentation.
   d) EPS PhD students day (2004), best poster prize.

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ORIGINAL PAPER

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The potato *P* locus codes for flavonoid 3′,5′-hydroxylase

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Abstract The potato *P* locus is required for the production of blue/purple anthocyanin pigments in any tissue of the potato plant such as tubers, flowers, or stems. We have previously reported, based on RFLP mapping in tomato, that the gene coding for the anthocyanin biosynthetic enzyme flavonoid 3′,5′-hydroxylase (*f3′5′h*) maps to the same region of the tomato genome as *P* maps in potato. To further evaluate this association a *Petunia f3′5′h* gene was used to screen a potato cDNA library prepared from purple-colored flowers and stems. Six positively hybridizing cDNA clones were sequenced and all appeared to be derived from a single gene that shares 85% sequence identity at the amino acid level with *Petunia f3′5′h*. The potato gene cosegregated with purple tuber color in a diploid F_1 subpopulation of 37 purple and 25 red individuals and was found to be expressed in tuber skin only in the presence of the anthocyanin regulatory locus *I*. A potato *f3′5′h* cDNA clone was placed under the control of a doubled CaMV 35S promoter and introduced into the red-skinned cultivar 'Désirée'. Tuber and stem tissues that are colored red in Désirée were purple in nine of 17 independently transformed lines.

Introduction

Anthocyanin coloration in plants can be influenced by many factors such as pigment composition (Lewis et al. 1998), the presence of ions (Naumann and Horst 2003), the presence of co-pigments (Mol et al. 1998), vacuolar pH (Yoshida et al. 1995), and cell shape (Noda et al. 1994).

In diploid potato three classical loci—*I*, *R*, and *P*—are known to be involved in coloration of potato tuber skin (Dodds and Long 1955, 1956). *R* and *P* encode basic factors required for the production of red pelargonidin or blue/purple petunidin-based anthocyanin pigments, respectively, while *I* is required for the tissue-specific expression of anthocyanin pigments in tuber skin (Dodds and Long 1955, 1956). *P* is known to be epistatic to *R* (Dodds and Long 1955). Thus tubers with genotype *I- Rr Pp* or *Ii rr P* have purple skin while those with genotype *I- R- pp* are red. Potatoes lacking a functional allele at *I* produce white tubers, irrespective of the alleles present at *R* and *P*. The diploid *I*, *R*, and *P* loci functionally mimic, and are likely to be allelic to, the tetraploid *D*, *R* and *P* loci, respectively (De Jong 1991), originally described by Salaman (1910).

R, *I*, and *P* have previously been localized to chromosomes 2, 10, and 11, respectively (Van Eck et al. 1993, 1994). De Jong et al. (2004) recently mapped 13 anthocyanin-related genes in tomato, a crop that shares extensive conservation of marker order with potato. Comparing map locations across these two species led to the hypotheses that *I*, *R*, and *P* encode a MYB transcription factor, dihydroflavonol 4-reductase (*dfr*), and flavonoid 3′,5′-hydroxylase (*f3′5′h*), respectively.

In this research we test the latter hypothesis more thoroughly, and now report that the potato *f3′5′h* gene cosegregates with *P*, is expressed in tuber skin only in the presence of *I*, and when expressed as a transgene in the red-skinned cultivar Désirée changes tuber skin color from red to purple.

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Materials and methods

Plant material

Diploid potato clones W5281.2 (female parent/purple tuber skin/genotype *Ii RR Pp*) (De Jong and Burns 1993)

and 07506-01 (male parent/white tuber skin/genotype *ii rr pp*) (De Jong 1987; De Jong and Burns 1993) were crossed to generate an F_1 population that segregates for tuber skin color (approximately 2 white:1 red:1 purple). The cultivar Désirée was obtained from the New York State Foundation Seed Farm, Lake Placid, N.Y.

cDNA library screening

A cDNA library from pigmented flowers and stems of W5281.2 has previously been constructed (De Jong et al. 2003b). A fragment of the *Petunia f3′5′h* gene, corresponding to the majority of the third exon, was amplified with primers 3Hf1-F (5′-TTTGTTCACAGCTGGTACGG) and 3Hf1-R (5′-AGAGGGACAGCTTTCTGCAA), radioactively labeled, and then used as a hybridization probe to screen the cDNA library. Positive clones were plaque-purified and the inserts then excised to permit propagation in a pBlueScript SK− phagemid vector, following the manufacturer's instructions. Inserts were sequenced using universal M13 vector primers.

Cloning and analysis of genomic DNA

One of the cDNA clones isolated from the potato library appeared to contain the entire open reading frame (ORF) of a gene with high sequence similarity (82% nucleotide identity) to *Petunia f3′5′h*. Primers 1F (5′-CGCGGCAATAATATACATCATAGT) and 1R (5′-GCAAGGGCCCATTCTATTACA) were designed to amplify the first three-quarters of the corresponding gene from genomic DNA. The resulting PCR products were cloned into the vector pGEM-T (Promega), sequenced, and examined for polymorphism. Two haplotypes were observed. To determine whether either cosegregated with purple skin color, primers 3F (5′-ATGAACGTGTGCAAAAGAAAACTC) and 3R (5′-AATTTATAGTACTTTTCGTATAGTTTTTGA) were designed to flank a polymorphic *Taq* I restriction site. Products were digested with *Taq* I after amplification, separated on a 1% agarose gel, and visualized by staining with ethidium bromide. Primers 2F (5′-GTCTGCTCTCTTAAGATATTGTTGTG) and 2R (5′-AATTACAAAACAAATCCCTTCAAG) were used to amplify the 3′ third of the potato gene, which was also cloned into pGEM-T and sequenced.

RT-PCR analysis of gene expression

Primers 4F (5′-GTTGTTGCCTCTACCCCTAAT) and 4R (5′-AACTTTTCATCCCTTTTTCAAT) flank a polymorphic *Cla* I restriction site and were used to study expression of potato *f3′5′h*. These primers flank an intron so that amplification products generated from genomic and cDNA templates can readily be distinguished. Products were digested with *Cla* I after amplification, separated on a 1% agarose gel, and visualized by staining with ethidium bromide. For each independent experiment evaluating *f3′5′h* gene expression total RNA was isolated with an RNeasy Plant Mini Kit (Qiagen) from the tuber skin of each parent (W5281.2 and 07506-01), two progeny with red tuber skin, two progeny with white skin, and two progeny with purple skin. The experiment was performed four times so that a total of eight red, eight white, and eight purple progeny were examined. RNA was isolated from young tubers, 1–3 cm in diameter. Total RNA from each clone was treated with RQ1 RNase-free DNase (Promega) to eliminate genomic DNA prior to reverse transcription. Treated total RNA was then converted into cDNA using random primers and a Reverse Transcription System kit (Promega). Approximately 70 ng of cDNA was then used as a template for PCR, employing the following thermal profile: 1 min at 94°C, followed by 35 cycles of 15 s at 94°C, 15 s at 53°C, and 1 min at 72°C, and a final 10 min incubation at 72°C. The same procedure was used to examine *f3′5′h* expression in leaves of transgenic plants, except that products were not digested with *Cla* I prior to electrophoresis.

Transformation construct

The complete potato *f3′5′h* ORF was amplified from a cDNA clone using a low error-rate polymerase (Platinum *Pfx* DNA polymerase, GIBCO-BRL) and primers F35HORF-XbaI-F (5′-GGG TCTAGAATGACGTTACGTATTAGTGAGTTGTTTGC) and F35HORF-SacI-R (5′-CCC GAGCTCTCAGCAACAATAAACGTCCAAAGATAG). The 5′ portions of the primers were extended to include *Xba* I and *Sac* I restriction sites, respectively (underlined). The products were digested with both enzymes and ligated into the *Xba* I and *Sac* I sites of transformation vector pPS1 (Huang and Mason 2004). This vector contains a doubled CaMV promoter and tobacco etch virus translational enhancer sequence to enhance transgene expression. The insert was sequenced to ensure that no mutations had been introduced during PCR and then the transformation construct was introduced into *Agrobacterium tumefaciens* strain LBA4404.

Potato transformation

Potato internode segments from 8-week-old in vitro-grown cultivar Désirée were cut into 0.5–1.0 cm explants, and then placed on callus induction medium (CIM) [4.3 $g.l^{-1}$ Murashige and Skoog salts, 26.64 μM glycine, 4.06 μM nicotinic acid, 2.43 μM pyridoxine HCl, 1.19 μM thiamine HCl, 0.57 μM folic acid, 0.20 μM biotin, 0.555 mM myo-inositol, 87.64 mM sucrose, 4.44 μM 6-benzylamino purine (BAP), 10.74 μM 1-naphthaleneacetic acid (NAA), and 2.5 $g.l^{-1}$ Phytagel (Sigma,

catalog no. P8169), with pH adjusted to 5.6] in plates. The explants were incubated in *Agrobacterium* inoculum for 10 min, agitating occasionally, removed from the inoculum, and blotted on sterile filter paper. The explants were then plated on CIM and incubated for 48 h in the dark at 19°C. These explants were then transferred to 3C5ZR medium [4.3 $g.l^{-1}$ Murashige and Skoog salts, 2.96 μM thiamine HCl, 4.06 μM nicotinic acid, 2.43 μM pyridoxine HCl, 0.555 mM myo-inositol, 87.6 mM sucrose, 2.85 μM 3-indoleacetic acid (IAA), 8.54 μM trans-zeatin-riboside, and 2.5 $g.l^{-1}$ Phytagel (Sigma), with pH adjusted to 5.9] containing 75 $mg.l^{-1}$ kanamycin monosulfate and 200 $mg.l^{-1}$ timentin (ticarcillin disodium and clavulanic acid) (GlaxoSmithKline, Research Triangle Park, N.C., USA), and then transferred to fresh 3C5ZR medium weekly for a month and every 10–14 days thereafter. After approximately 8 weeks regenerated shoots (1.5 cm long) were excised and transferred into shoot propagation/rooting medium (CM) (4.3 $g.l^{-1}$ Murashige and Skoog salts, 1.19 μM thiamine HCl, 0.555 mM myo-inositol, 58.43 mM sucrose, and 2.5 $g.l^{-1}$ Phytagel, with pH adjusted to 5.7) containing kanamycin and timentin. After 1 month, shoots were transferred to fresh CM containing timentin and no kanamycin. After another month they were transferred to fresh CM without timentin and then monitored for growth of *Agrobacterium* to ensure that the plants were free of bacteria.

Thin layer chromatography

Approximately 0.1 g of tuber skin was macerated in a 1.5 ml tube containing 200 µl of 99% ethanol/1% HCl (v:v) and then centrifuged to pellet insoluble material. Repeated applications of 5 µl aliquots were spotted onto cellulose thin layer chromatography plates (Eastman Kodak 6064), allowed to dry, and then developed with butanol/2 N HCl (1:1 v:v) (Dodds and Long 1955).

Characterization of *f3′5′h* cDNA in a red-skinned cultivar

Primers F35Hexon1F (5′- ATGACGTTACGTATTAGTGAGTTG) and F35Hexon3R (5′- TCAGCAACAATAAACGTCCAAA) anneal to the beginning and end of the *f3′5′h* ORF, respectively; bases corresponding to the start and stop codons are underlined. These primers were used to amplify cDNA prepared as described above from tuber skin of the cultivar 'Rideau'. The PCR products were sequenced directly without cloning.

Results

Isolation of a candidate gene for *P*

A potato cDNA library, prepared from purple flowers and stems of the diploid potato clone W5281.2 (genotype *Ii RR Pp;* De Jong and Burns 1993), was screened using a PCR product corresponding to most of the third exon of *Petunia f3′5′h* as a hybridization probe. Six independent cDNA clones were isolated and partially sequenced. All appeared to be derived from the same gene, and likely the same allele, as no sequence polymorphism was observed. One cDNA clone contained the entire ORF and was sequenced in its entirety (GenBank accession AY675558). Potato *f3′5′h* is predicted to encode a protein of 509 amino acids and share 85% amino acid sequence identity with *Petunia f3′5′h* (GenBank accession P48419) (Fig. 1). The gene product contains a cytochrome P450 domain with the conserved group A signature motif PFGXGRRXCXG (Durst and Nelson 1995) at amino acids 438 through 448.

Corresponding genomic DNA of W5281.2 was characterized by amplifying, cloning, and sequencing two overlapping fragments of the gene, using the cDNA sequence as a guide for primer design. The first

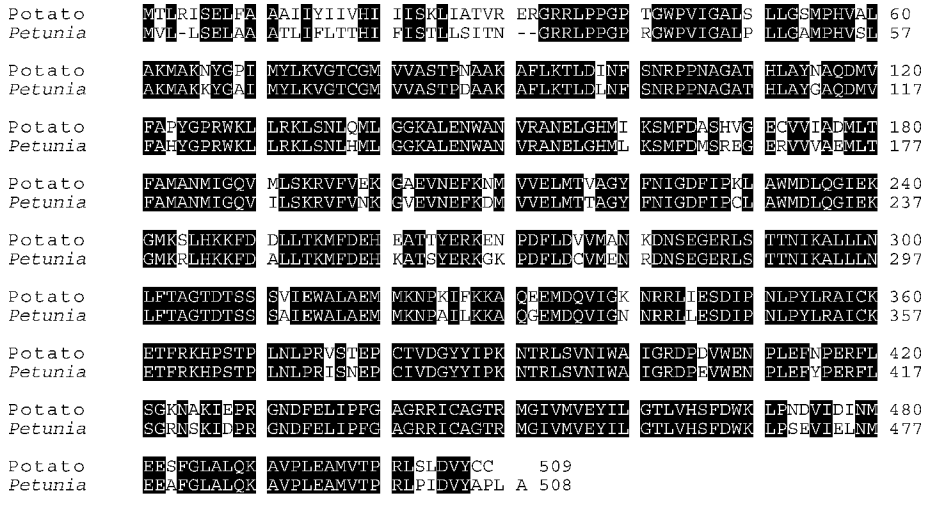


Fig. 1 Alignment of potato and *Petunia f3′5′h* gene products. Identical amino acids are shown with *white type* and *black highlighting*, while non-conserved amino acids are shown with *black type on a white background*

fragment evaluated corresponded to the first three-quarters of the gene. Cloning and sequencing this region revealed two alleles that shared 96% sequence identity, indicating that W5281.2 is heterozygous for *f3′5′h*. The predicted exons of one of these two alleles matched the original cDNA sequence perfectly. When amplification products corresponding to the 3′ third of the gene were cloned and sequenced only one allele—that corresponding to the original cDNA sequence—was observed. The same result occurred when several alternative primers in the 3′ untranslated region and 3′ end of the third exon were used, suggesting considerable sequence polymorphism between the two alleles in this region. The structure of the allele corresponding to the original cDNA sequence is shown in Fig. 2a. Like *Petunia* the potato *f3′5′h* gene contains three exons and two introns.

Cosegregation and appropriate tissue expression

To investigate the relationship between potato *f3′5′h* and *P* we first asked whether either allele of *f3′5′h* cosegregated with purple skin color. An F_1 population that segregated 2:1:1 for white:purple:red tuber skin color was constructed by crossing W5281.2 with the white-skinned clone 07506-01 (*ii rr pp*). Using a polymorphic *Taq* I restriction site in the second intron (Fig. 2a) to differentiate haplotypes revealed that the allele corresponding to the original cDNA sequence absolutely cosegregated with purple skin color among the 37 purple-skinned and 25 red-skinned progeny tested (Fig. 2b).

The expression of potato *f3′5′h* was then examined in tuber skin of W5281.2 and 07506-01, as well as in selected progeny, by RT-PCR (Fig. 3). Both alleles of the gene were found to be expressed in W5281.2, and at least one allele was expressed in all purple and red-skinned progeny, i.e., all progeny that carry a dominant allele at the regulatory locus *I*. The gene was not expressed in 07506-01 or in any white progeny. This result is in accordance with our recent finding (C.S. Jung et al. unpublished) that *I* is a transcription factor that coordinately regulates the expression of multiple anthocyanin pathway genes. In addition to the polymorphic *Taq* I site described above, a polymorphic *Cla* I restriction site within the second exon also differentiates the two alleles of *f3′5′h* in W5281.2 (Fig. 2a). The *Cla* I site is absent in the allele linked in coupling with *P*. RT-PCR followed by *Cla* I digestion revealed that this allele was expressed in progeny clones with purple tuber skin (Fig. 3).

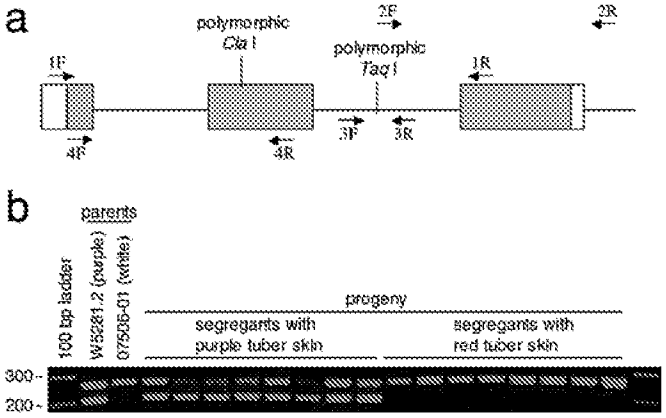


Fig. 2 Structure of the potato *f3′5′h* gene and cosegregation of this gene with purple tuber skin color. **a** The potato *f3′5′h* gene contains three exons (indicated with *open boxes*) and two introns (indicated with *solid lines*). Regions with sequence similarity to a domain conserved in cytochrome P450 proteins (Marchler-Bauer et al. 2003) are highlighted in *gray*. Primer binding sites are indicated with *arrows*. Primer pairs 1F + 1R and 2F + 2R were used to clone and sequence the gene (GenBank accessions AY675559, AY675560). Primer pairs 3F + 3R and 4F + 4R flank polymorphic *Taq* I and *Cla* I restriction sites, respectively, and were used to differentiate the two alleles of this gene in diploid W5281.2. One W5281.2 allele possesses a *Taq* I restriction site but lacks a *Cla* I restriction site, while the other lacks the *Taq* I site but can be cleaved with *Cla* I. **b** Products generated with primers 3F + 3R were digested with *Taq* I and then electrophoresed on a 1% agarose gel. The W5281.2 *f3′5′h* haplotype defined by the presence of the *Taq* I restriction site yields a 210 bp fragment with this assay; this fragment was observed to cosegregate with purple tuber color. The *left lane* contains a 100 bp size ladder; the length of two marker bands is indicated at *left*

Complementation

The ORF of the potato *f3′5′h* allele linked in coupling with *P* was placed into an *Agrobacterium* binary vector under the control of a doubled CaMV 35S promoter and introduced into the red-skinned cultivar Désirée. Nine of 17 plants transformed with this construct displayed purple stems and purple tubers, while the remaining eight plants displayed only pale red tubers and stems that were indistinguishable in color from untransformed Désirée as well as control plants transformed with empty vector (Fig. 4 and data not shown). Except for the difference in color, transformed and

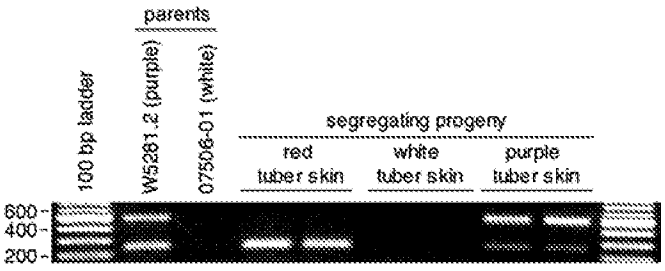


Fig. 3 Expression of potato *f3′5′h* in tuber skin. Primers 4F and 4R (Fig. 2a) were used to amplify cDNA prepared from tuber skin of parents W5281.2 and 07506-01 as well as from two progeny with red skin, two progeny with white skin, and two progeny with purple skin. PCR products were digested with *Cla* I and electrophoresed on a 1% agarose gel to discriminate the alleles expressed

untransformed plants were otherwise phenotypically indistinguishable.

Thin layer chromatography showed that the anthocyanin pigment profile in transformed Désirée tubers with the purple phenotype was indistinguishable from that of W5281.2, but clearly distinct from that of untransformed Désirée (Fig. 4). RT-PCR analyses indicated that at least one allele of *f3′5′h* is expressed in Désirée leaf tissue (Fig. 5). Accumulation of *f3′5′h* transcripts was noticeably higher in transgenic plants with purple tubers and stems, while *f3′5′h* levels in transformed plants that showed no purple color were comparable to untransformed Désirée (Fig. 5).

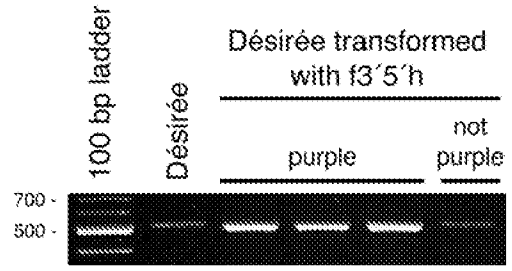


Fig. 5 RT-PCR expression analysis of the *f3′5′h* transgene. Total RNA was isolated from leaves of Désirée and of Désirée transformed with *f3′5′h*. RNA was treated with DNase to eliminate genomic DNA, converted into cDNA, and then subjected to PCR with primers 4F and 4R (Fig. 2a). Amplification products were separated on a 2% agarose gel. Transformed Désirée plants included those with a purple tuber phenotype, as well as those indistinguishable from wild type (*not purple*)

Sequence of a transcribed, non-functional allele of *f3′5′h*

The RT-PCR analyses of Figs. 3 and 5 revealed that *f3′5′h* is transcribed in at least some plants with red tuber skin. Any *f3′5′h* alleles expressed in tissue with red anthocyanin presumably carry one or more mutations that interfere with protein function. To determine whether this was indeed the case we characterized an *f3′5′h* allele expressed in the red-skinned cultivar 'Rideau'. Primers designed to amplify the entire ORF of the functional allele from W5281.2 directed amplification of a 1,530 bp product from Rideau tuber skin cDNA. The sequence trace of the uncloned Rideau PCR product (GenBank accession AY675561) showed no mixed bases indicating that only one allele had been amplified. The predicted amino acid sequence of this allele differed at ten residues from the functional allele of W5281.2 (data not shown). One of the ten changes was within the

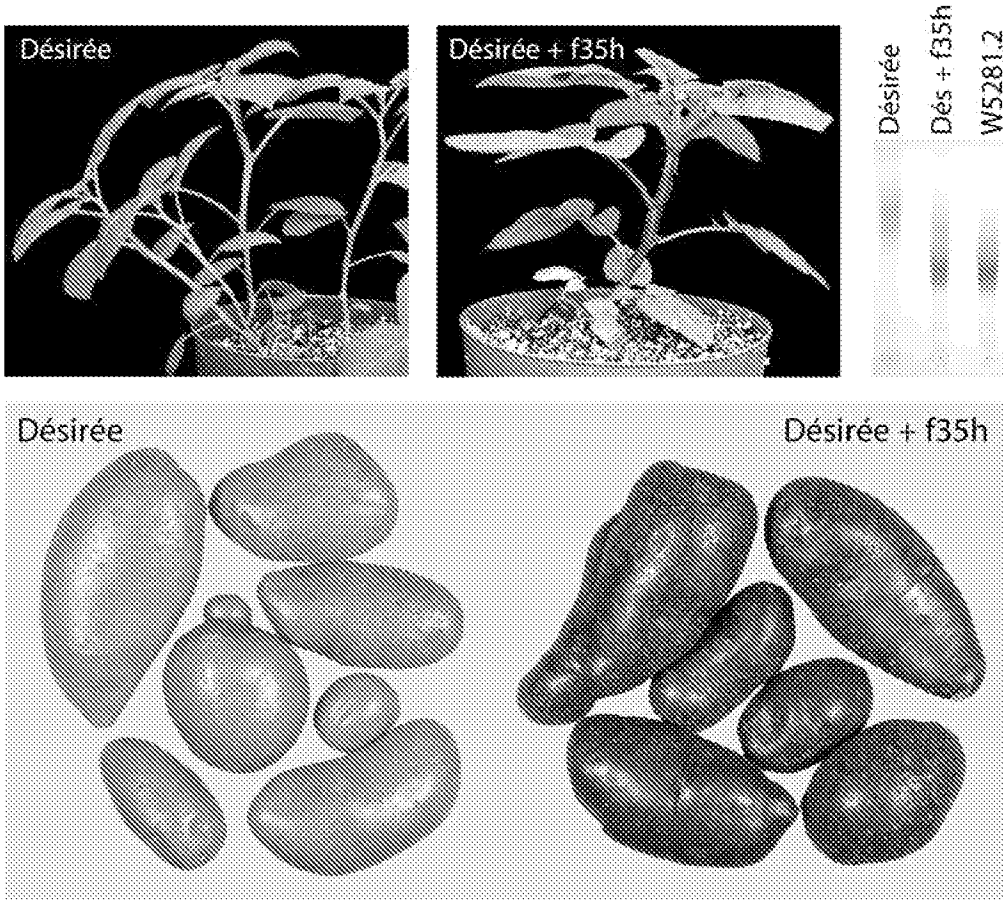


Fig. 4 Transformation of the red-skinned cultivar Désirée with potato *f3′5′h*. Foliage and tubers of Désirée and a representative Désirée line transformed with *f3′5′h* are shown. *Top right*: thin layer chromatography of anthocyanin pigments isolated from tuber skin of untransformed Désirée, Désirée transformed with *f3′5′h*, and W5281.2. Pigment samples were applied near the bottom of the TLC plate shown and developed with a solvent comprising butanol/2 N HCl. The orange color and faster migration of the sample prepared from Désirée is characteristic of derivatives of pelargonidin, while the purple color and slower migration in the remaining two lanes is characteristic of derivatives of petunidin (Dodds and Long 1955)

conserved group A P450 signature motif PFGXGRRXCXG (Durst and Nelson 1995). The sequence was PFGAGRRICAG in W5281.2 and PFWAGRRICAG in Rideau.

Discussion

The *P* locus has long been known to be required for the production of blue/purple anthocyanins in potato (Dodds and Long 1955, 1956). The observations that *f3′5′h* is required for blue/purple pigment production in *Petunia* (Holton et al. 1993) and that *f3′5′h* and *P* map to similar regions of the genome (De Jong et al. 2004) led us to hypothesize that the potato homolog of *f3′5′h* would correspond to *P*. To test this we isolated an *f3′5′h* cDNA clone from a purple-skinned potato using the *Petunia* gene as a hybridization probe. The corresponding gene was found to cosegregate with purple tuber skin color and was expressed in tuber skin only in the presence of the anthocyanin regulatory locus *I*. When the potato cDNA clone was transformed into the red-skinned cultivar Désirée, tissues that were normally red in color were now purple. The genetic cosegregation, biologically relevant transcriptional regulation, and complementation data collectively provide strong evidence that the potato *f3′5′h* gene corresponds to *P*.

Identification of the gene underlying *P* provides opportunities to more efficiently manipulate color in potato breeding programs through marker assisted selection or transgenic approaches. It is often possible to determine whether a given potato clone has a functional allele of *P* simply by looking for the presence of purple pigment in tubers, sprouts, stems, or flowers. Visual inspection cannot, however, reveal dosage (*Pppp*, *PPpp*, *PPPp*, *PPPP*) of the functional allele. Parental clones with higher dosage transmit the corresponding trait to a higher proportion of progeny, and are thus preferred when breeding. It may now be possible to develop an assay to measure dosage of desirable alleles at this locus, e.g., by utilizing a 5′ fluorogenic nuclease approach, as has been done with *dfr* (De Jong et al. 2003a). As demonstrated in this work it is also possible to convert a red-skinned cultivar into a purple-skinned derivative by transformation with a functional allele. The same approach would likely convert a red-fleshed cultivar into a purple-fleshed derivative. Converting white-skinned clones into purple-skinned clones will presumably require co-transformation with a functional allele of *I*. With appropriate promoters driving tissue-specific expression (or silencing) of *P* it may also be possible to create novel bi-color patterns as well, e.g., a red-skinned tuber with purple flesh.

Lewis et al. (1998) have previously shown that anthocyanin pigments in tubers of red cultivars are derived primarily from pelargonidin, while in purple clones they are derived mostly from petunidin. Dodds and Long (1955) reported that *P* is epistatic to *R*; the pelargonidin pigments present in tubers with genotype *I- R- pp* are to a large extent replaced with petunidins in tubers with genotype *I- R- P-*. Dodds and Long (1955) also observed in plants with both *R* and *P* that as the concentration of pelargonidin went up, that of petunidin was reduced, and vice versa. Epistasy of *P* to *R* was thus suspected to occur as a result of competition for a common precursor (Dodds and Long 1955). De Jong et al. (2003b) have recently presented evidence that the *R* locus codes for dihydroflavonol 4-reductase (*dfr*). Consistent with the early inference by Dodds and Long (1955), the gene products of *dfr* and *f3′5′h* are thought to compete for the common precursor dihydrokaempferol (Holton and Cornish 1995).

At least one allele of *f3′5′h* was found to be expressed in the red-skinned cultivars Désirée and Rideau, as well as in the red-skinned progeny of the cross between W5281.2 and 07506-01. In all these cases the difference between dominant (functional) and recessive (non-functional) alleles at *P* thus appears to result from mutations that affect protein function rather than mutations that affect gene transcription. Sequencing the allele expressed in Rideau revealed ten amino acid differences compared to the functional allele of W5281.2, including one within a highly conserved motif. The latter mutation is a promising candidate for explaining why the Rideau allele is non-functional, although a role for one or more of the other mutations cannot be ruled out.

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ORIGINAL PAPER

The potato *developer* (*D*) locus encodes an R2R3 MYB transcription factor that regulates expression of multiple anthocyanin structural genes in tuber skin

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Abstract A dominant allele at the *D* locus (also known as *I* in diploid potato) is required for the synthesis of red and purple anthocyanin pigments in tuber skin. It has previously been reported that *D* maps to a region of chromosome 10 that harbors one or more homologs of *Petunia an2*, an R2R3 MYB transcription factor that coordinately regulates the expression of multiple anthocyanin biosynthetic genes in the floral limb. To test whether *D* acts similarly in tuber skin, RT-PCR was used to evaluate the expression of flavanone 3-hydroxylase (*f3h*), dihydroflavonol 4-reductase (*dfr*) and flavonoid 3′,5′-hydroxylase (*f3′5′h*). All three genes were expressed in the periderm of red- and purple-skinned clones, while *dfr* and *f3′5′h* were not expressed, and *f3h* was only weakly expressed, in white-skinned clones. A potato cDNA clone with similarity to *an2* was isolated from an expression library prepared from red tuber skin, and an assay developed to distinguish the two alleles of this gene in a diploid potato clone known to be heterozygous *Dd*. One allele was observed to cosegregate with pigmented skin in an F_1 population of 136 individuals. This allele was expressed in tuber skin of red- and purple-colored progeny, but not in white tubers, while other parental alleles were not expressed in white or colored tubers. The allele was placed under the control of a doubled 35S promoter and transformed into the light red-colored cultivar Désirée, the white-skinned cultivar Bintje, and two white diploid clones known to lack the functional allele of *D*. Transformants accumulated pigment in tuber skin, as well as in other tissues, including young foliage, flower petals, and tuber flesh.

Abbreviations

dfr	Dihydroflavonol 4-reductase
f3′5′h	Flavonoid 3′,5′-hydroxylase
f3h	Flavanone 3-hydroxylase
Stan2	*Solanum tuberosum* anthocyanin 2
Stan3	*Solanum tuberosum* anthocyanin 3

Introduction

Most natural variation in potato tuber color results from tissue-specific differences in the accumulation of anthocyanin pigments. An early study by Salaman (1910) in tetraploid potato (*Solanum tuberosum* L.) identified three loci—*D* (developer), *R* (red), and *P* (purple) that condition variation for color of potato tuber skin. *R* is required for the production of red anthocyanins, *P* is required for the production of purple anthocyanins, while *D* is required for the tissue-specific accumulation of anthocyanin pigments in tuber skin. Red and purple tuber anthocyanins have been shown to be derivatives of pelargonidin and delphinidin, respectively (Lewis et al. 1998; Naito et al. 1998; Rodriguez-Saona et al. 1998).

A parallel three-gene system, but using the gene symbols *I* (inhibitor), *R*, and *P*, was subsequently described in diploid potato by Dodds and Long (1955, 1956). With diploids *P* was shown to be epistatic to *R*. Thus, tubers with genotype *I*– *R*– *P*– or *I*– *rr P*– have purple tuber skin, those with genotype *I*– *R*– *pp* have red colored skin, and those with genotype *ii* – – are white. The similar phenotypes mediated by tetraploid *D*, *R*, and *P* and diploid *I*, *R*, and *P* imply that *D* is equivalent to *I*, tetraploid *R* is equivalent to diploid *R*, and tetraploid *P* is equivalent to diploid *P*

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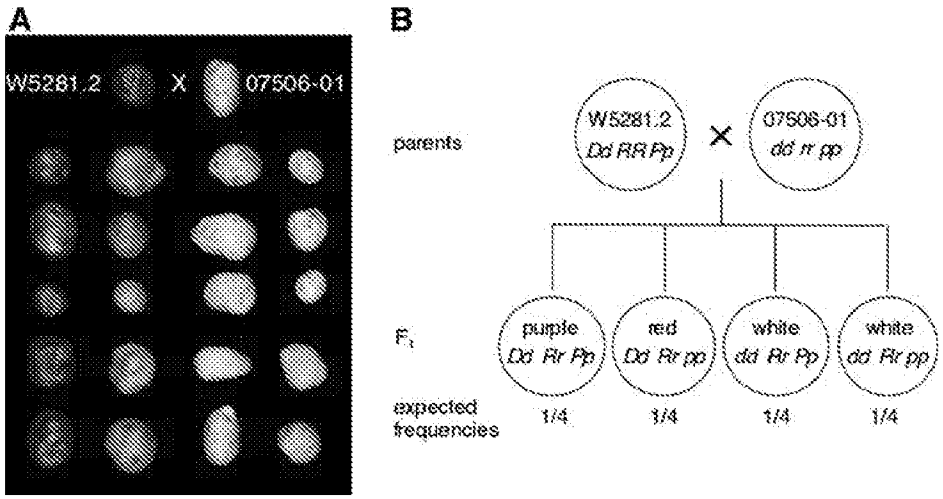


Fig. 1 Diploid population segregating for tuber skin color and schematic diagram illustrating allele segregation. **a** A cross between W5281.2 (female/purple tuber skin/genotype *Dd RR Pp*) and 07506-01 (male/white tuber skin/genotype *dd rr pp*) generated an F_1 population that segregated for tuber skin color (76 purple:72 red:132 white). Parents are shown at the top, 20 representative progeny are shown below. **b** Schematic diagram of the F_1 population showing parental and progeny genotypes and their predicted frequencies. *D* is required for color expression in tuber skin (red or purple), while *R* and *P* are required for red and purple color expression, respectively. Purple color is epistatic to red

(reviewed in De Jong 1991). To provide consistency, the original gene nomenclature of Salaman (1910) will be used throughout this paper.

D, *R*, and *P* (*sensu* Salaman) have previously been localized to chromosomes 10, 2, and 11, respectively (Gebhardt et al. 1989; van Eck et al. 1993, 1994). Subsequent mapping of many anthocyanin structural and regulatory genes in tomato gave rise to the suggestion that *R* corresponds to dihydroflavonol 4-reductase (*dfr*), that *P* codes for flavonoid 3′,5′-hydroxylase (*f3′5′h*), and that *D* encodes an R2R3 MYB transcription factor similar to *Petunia an2* (De Jong et al. 2004). A combination of genetic cosegregation and transgenic evidence has confirmed that *R* encodes *dfr* (De Jong et al. 2003a; Zhang et al. 2009b) and that *P* encodes *f3′5′h* (Jung et al. 2005). R2R3 MYB genes have been shown to coordinately regulate the expression of multiple anthocyanin structural genes in several species, including maize, apple, and grape (Cone et al. 1986; Espley et al. 2007; Cutanda-Perez et al. 2009).

With this paper, we report that *D* is a transcriptional regulator of the anthocyanin biosynthetic pathway in tuber periderm and identify an R2R3 MYB domain gene that cosegregates with *D* and is expressed in red and purple, but not white, tuber skin.

Materials and methods

Plant material

Diploid potato clones W5281.2 (female parent/purple tuber skin/genotype *Dd RR Pp*) (De Jong and Burns 1993) and 07506-01 (male parent/white tuber skin/genotype *dd rr pp*) (De Jong 1987; De Jong and Burns 1993) were crossed to generate an F_1 population that segregates for tuber skin color (2 white:1 red:1 purple) (Fig. 1). Breeding clones with an NY designation were developed by the Cornell University potato breeding program. The tetraploid Cornell breeding clone Y83-5 (red tuber skin/white tuber flesh) was used to construct a cDNA library. In vitro plantlets of the cultivars Désirée and Bintje were obtained from the New York State Seed Farm in Lake Placid, NY, USA and the Potato Introduction Station in Sturgeon Bay, WI, USA, respectively.

cDNA library construction and screening

Total RNA was isolated from tuber skin of Y83-5 with a LiCl-based procedure (De Vries et al. 1988). Messenger RNA was then purified from total RNA with a PolyTract mRNA isolation system kit (Promega). Double stranded cDNA was synthesized with a ZAP cDNA synthesis kit (Stratagene) and cloned into a lambda DASH II vector (Stratagene). A fragment of the *Petunia an2* gene was amplified with primers an2–5′ and an2–3′ (Quattrocchio et al. 1998), radioactively labeled, and then used as a probe to screen the Y83-5 cDNA library, as well as a cDNA library previously constructed from diploid W5281.2 (De Jong et al. 2003a). Hybridizing clones were plaque-purified and the inserts then excised to permit propagation in a pBlueScript SK(−) phagemid vector, following the manufacturer's instructions. Inserts were sequenced using universal M13 vector primers.

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Cloning and analysis of genomic DNA

Primers Stan2-F4 (5′GTAATAAGGAAAGGTTCATGGACT) and Stan2-R (5′AAAATTCATCCCAACCACCATCAC) were designed against the predominant sequence class (*Stan2*) isolated from the Y83-5 cDNA library and used to amplify the majority of the corresponding gene from W5281.2. The resulting PCR products were cloned into the vector pGEM-T (Promega), sequenced, and examined for polymorphism. Two haplotypes were observed. To determine whether either haplotype cosegregated with tuber skin color, primers Stan2-ClaI-F2 (5′GTGATTATGTCATCCAAAAGTTTATAG) and Stan2-ClaI-R1 (5′GAATTTCTGAGGTTGAGGTCTTA) were designed to flank a polymorphic *Cla* I restriction site within the second intron. Products were digested with *Cla* I after amplification, separated on a 1% agarose gel, and visualized by staining with ethidium bromide. The same primers were used to evaluate the allelic composition of tetraploid cultivars and breeding clones. Here, primer Stan2-ClaI-F2 was radioactively labeled with ^{33}P prior to PCR, and undigested products were separated on a 5% denaturing acrylamide sequencing gel.

Primers Stan3-F1 (5′GGTTCATGGACTGAACAAGAAGATTT) and Stan3-R1 (5′ATTCAGTCAGATTCTAATATAAGTACC) were designed against the less abundant cDNA sequence class (*Stan3*) and used to amplify the majority of one allele from W5281.2. Comparing the sequence of this allele to the cDNA previously isolated from Y83-5 revealed a polymorphic $(CTC)_n$ simple sequence repeat in the third exon. Primers Stan3-F4 (5′CAGCAAACGATGTGAAGAAC) and Stan3-R4 (5′TGTAGATGAGGATTAGGACGAG) were designed to flank this repeat and then used to test whether either *Stan3* alleles of W5281.2 cosegregated with tuber skin color.

RT-PCR analysis of gene expression

A schematic diagram of part of the anthocyanin biosynthetic pathway, illustrating metabolic intermediates and enzymes, is presented in Fig. 2. Primers F3H-F1 (5′AACAAGTTTTATTAGGGATGAA) and F3H-R1 (5′CACGCCAGTCTTGAACCA) were designed against conserved regions of two published potato flavanone 3-hydroxylase (*f3h*) cDNA sequences (GenBank accessions AY102035 and BE339992). Primers potDFR1 and potDFR2 amplify an internal fragment of dihydroflavonol 4-reductase (*dfr*) and have been described previously (De Jong et al. 2003a). Primers F35H-F2 (5′GTTGTTGCCTCTACCCCTAAT) and F35H-R2 (5′AACTTTTCATCCCTTTTTCAAT) were designed against the sequence of a potato cDNA clone isolated from a W5281.2 cDNA library using *Petunia* flavonoid 3′,5′-hydroxylase (*f3′5′h*) as a probe (Jung et al. 2005). Primers Stan2-F1 (5′GAGAGGTGACTTTGATTG) and Stan2-R1 (5′CAGTATATTTGCCCACCATTG) were used to study expression of *Stan2*. Primers Stan3-F2 (5′GAGACTTCATAAACTCTTAGGCAATAG) and Stan3-R2 (5′TATGTAGATGAGGATTAGGACGAG) were used to study expression of *Stan3*. All five primer pairs flank an intron so that amplification products generated from genomic and cDNA templates can readily be distinguished. Primers 18S-F1 (5′TGCCAGTAGTCATATGCTTGTCTC) and 18S-R1 (5′AGCCCGGTATTGTTATTTATTGTC) were designed against potato 18S ribosomal RNA. This primer pair was used to control for the success of RT-PCR when characterizing W5281.2 and 07506-01 and their progeny. When evaluating expression of *Stan2* in tuber skin of selected cultivars and breeding clones, a QuantumRNA™ 18S internal standard (Ambion) was used to control for the success of RT-PCR, following the manufacturer's instructions.

For each independent experiment evaluating anthocyanin structural gene expression, total RNA was isolated with an RNeasy Plant Mini Kit (Qiagen) from the tuber skin (the surface tissue that could be peeled off with forceps) of parents W5281.2 and 07506-01, two progeny with red skin, two progeny with white skin, and two progeny with purple skin. The identity of the red-, white- and purple-skinned progeny varied between experiments. The experiment was performed four times, so that a total of eight red, eight white, and eight purple progenies were examined. RNA was isolated from young tubers, one to three cm in diameter, from plants that had not yet senesced. Total RNA from each clone was treated with RQ1 RNase-free DNase (Promega) to eliminate genomic DNA prior to reverse transcription. RNA was then converted into cDNA using random primers and a reverse transcription system kit (Promega). Approximately 70 ng of cDNA was then used as a template for PCR, employing the following thermal profile: 1 min at 94°C, followed by 35 cycles of [15 s at 94°C, 15 s at 53°C, and 1 min at 72°C], and a final 10 min incubation at 72°C.

Testing which alleles of Stan2 are expressed

Primers Stan2-F4 and Stan2-ClaI-R1 were used to amplify a fragment containing a polymorphic *Alu* I restriction site to determine which *Stan2* alleles of W5281.2 and 07506-01 are expressed in the skin of colored progeny. Complementary DNA, prepared in the same way as for the RT-PCR analyses described above, was amplified from a total of eight red-skinned and seven purple-skinned progenies. PCR products were then digested with *Alu* I.

Transformation

A *Stan2*^{777} cDNA isolated from W5281.2 (GenBank accession AY841129) appeared to contain all but the first two

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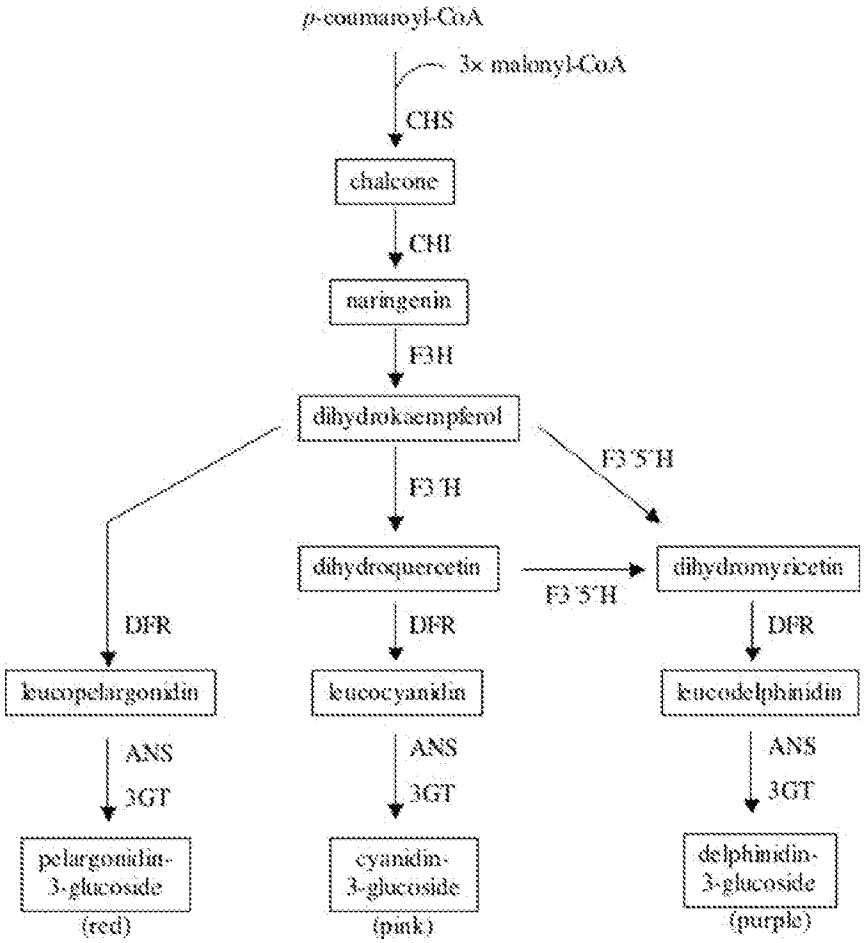


Fig. 2 Schematic diagram of the anthocyanin biosynthetic pathway, adapted from Holton and Cornish (1995). Condensation of one molecule of *p*-coumaroyl CoA with three molecules of malonyl CoA is catalyzed by chalcone synthase. The chalcone is then processed along the pathway in a series of enzymatic reactions to form three basic types of anthocyanins—pelargonidin-3-glucoside, cyanidin-3-glucoside, and delphinidin-3-glucoside—after which varying additional modifications ultimately give rise to red, pink, and purple anthocyanin pigments, respectively. Some of the genes encoding pathway enzymes are: *chs* (chalcone synthase), *chi* (chalcone isomerase), *f3h* (flavanone 3-hydroxylase), *f3′h* (flavonoid 3′-hydroxylase), *f3′5′h* (flavonoid 3′,5′-hydroxylase), *dfr* (dihydroflavonol 4-reductase), *ans* (anthocyanidin synthase), and *3gt* (flavonoid 3-glucosyltransferase). Metabolic intermediates are shown in boxes

codons of the open reading frame when compared to *Stan2*^{816}. The *Stan2*^{777} cDNA was amplified with primers 777 *Sac* I adaptor F (5′GAGCTCatgagtACTCCTATGATG TGTA) and 777 *Sac* I adaptor R (5′GAGCTCCTAATTAA GTAGATTCCATATATC) using a low error-rate polymerase (Platinum Pfx DNA polymerase; GIBCO-BRL). The 5′ portions of the primers were extended to include *Sac* I restriction sites (underlined). The forward primer also added the missing two codons (lowercase) to the open reading frame. Amplification products were digested with *Sac* I and ligated into the *Sac* I site of transformation vector pPS1 (Huang and Mason 2004). This vector contains a doubled CaMV promoter and tobacco etch virus translational enhancer sequence to enhance transgene expression. The construct was partially sequenced to ensure that no undesirable mutations had been introduced during PCR and confirm that the insert was in the desired sense orientation. The transformation construct was then introduced into *Agrobacterium tumefaciens* strain LBA4404. Transformation of the cultivar Désirée, the cultivar Bintje, and two diploid progeny (*dd Rr pp* and *dd Rr Pp*) from a cross between W5281.2 and 07506-01 was performed as previously described (Jung et al. 2005).

Thin layer chromatography

Approximately 0.1 g of tuber skin was macerated in a 1.5 mL tube containing 200 μL of 99% ethanol/1% HCl (v:v) and then centrifuged to pellet insoluble material. Repeated applications of 1 μL aliquots were spotted onto cellulose thin layer chromatography plates (Eastman Kodak 6064), allowed to dry, and then developed with butanol/2 N HCl (1:1 v:v) (Dodds and Long 1955).

Sequence alignment

Multiple sequence alignment was performed with MegAlign software (Lasergene, DNAStar, Inc.).

Results

Coordinate regulation of three anthocyanin structural genes in tuber skin

The potato *D* locus is required for the tissue-specific accumulation of red or purple anthocyanins in tuber skin.

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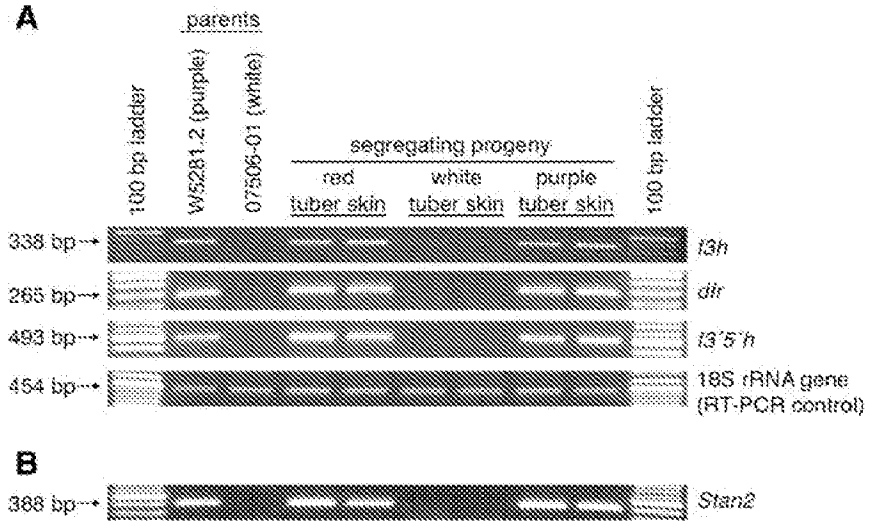


Fig. 3 Expression patterns of three anthocyanin structural genes (*f3h, dfr*, and *f3′5′h*) as well as *Stan2*, a candidate gene for *D*. **a** Expression of three anthocyanin structural genes in tuber periderm was examined by RT-PCR in parents W5281.2 and 07506-01 as well as in two progeny with red skin, two progeny with white skin, and two progeny with purple skin. Expression of 18S rRNA was evaluated as a control for RT-PCR. PCR product size and the corresponding gene name are denoted on the left and right of each gel, respectively. **b** Expression of *Stan2*, a candidate gene for the *D* locus, was examined by RT-PCR in the same parents and progeny as in **a**

Several genes that control similar tissue-specific accumulation of anthocyanins in model plant systems (*Petunia*, maize, *Arabidopsis*) are known to be transcription factors, which act by coordinately regulating the expression of multiple structural genes (Holton and Cornish 1995; Mol et al. 1998). Diploid W5281.2 has purple tuber skin and is heterozygous *Dd*, while diploid 07506-01 has white skin and is homozygous recessive *dd* (Fig. 1). Progeny of a cross between W5281.2 and 07506-01 segregate 1:1 for the presence or absence of anthocyanins in tuber skin (Fig. 1). To test the hypothesis that *D* encodes a transcription factor, we examined the expression of three anthocyanin structural genes—*f3h*, *dfr*, and *f3′5′h*—in the tuber skin of W5281.2 and 07506-01, as well as in selected progeny, by RT-PCR (Fig. 3a). All three structural genes were found to be expressed in W5281.2 and in all colored progeny, while no expression of *dfr* and *f3′5′h*, and only weak expression of *f3h*, were detected in 07506-01 or in any white progeny.

Isolation of candidate genes for D and cosegregation with tuber skin color

The *Petunia* R2R3 MYB domain gene *an2* coordinately regulates the expression of multiple anthocyanin genes in the floral limb (Quattrocchio et al. 1993). Tomato harbors at least two tightly linked homologs of *an2* that map to the same region of chromosome 10 where *D* is located in potato (De Jong et al. 2004). Reasoning that potato homolog(s) of *an2* expressed in tuber skin might correspond to *D*, a cDNA library constructed from tuber skin of Y83-5, a red-colored tetraploid breeding clone, was screened with an *an2* probe. Thirteen positively hybridizing clones were isolated and sequenced. Eleven cDNA clones were derived from one gene, which we designated *Stan2* for *Solanum tuberosum* anthocyanin 2, while the other two cDNA clones were derived from a second gene, which we designated *Stan3*. One of the *Stan2* cDNA clones (sequence deposited as GenBank accession AY841127) appeared, based on comparison with *an2*, to code for the entire open reading frame and was predicted to encode a protein of 271 amino acids. An interesting feature of the Y83-5 *Stan2* allele (hereafter designated $Stan2^{816}$) was a perfect duplication of 39 nucleotides (coding for 13 amino acids) in the third exon (Fig. 4). Neither of the *Stan3* cDNA clones were full-length (partial cDNA sequence has been deposited as accession AY841131). The predicted gene products of both *Stan2* and *Stan3* share extensive sequence similarity in their amino-terminal regions with several other known regulators of the anthocyanin pathway, including potato *StMtf1* (Rommens et al. 2008), Petunia *an2* (Quattrocchio et al. 1999, GenBank accession AF146702), tomato *ANT1* (Mathews et al. 2003), and pepper *A* (Borovsky et al. 2004) (Fig. 4). Sequence conservation is most evident in the amino-terminal R2 and R3 MYB domains. Relatively few amino acid residues are absolutely conserved in the second half of these proteins (Fig. 4).

Subsequent RT-PCR analyses with RNA template isolated from 13 cultivars revealed that *Stan2* was expressed in the skin of red-colored, but not white-colored, cultivars (Fig. 5). In addition, *Stan2* was not expressed in the tuber flesh of any of the same 13 clones (data not shown). *Stan3*, on the other hand, was

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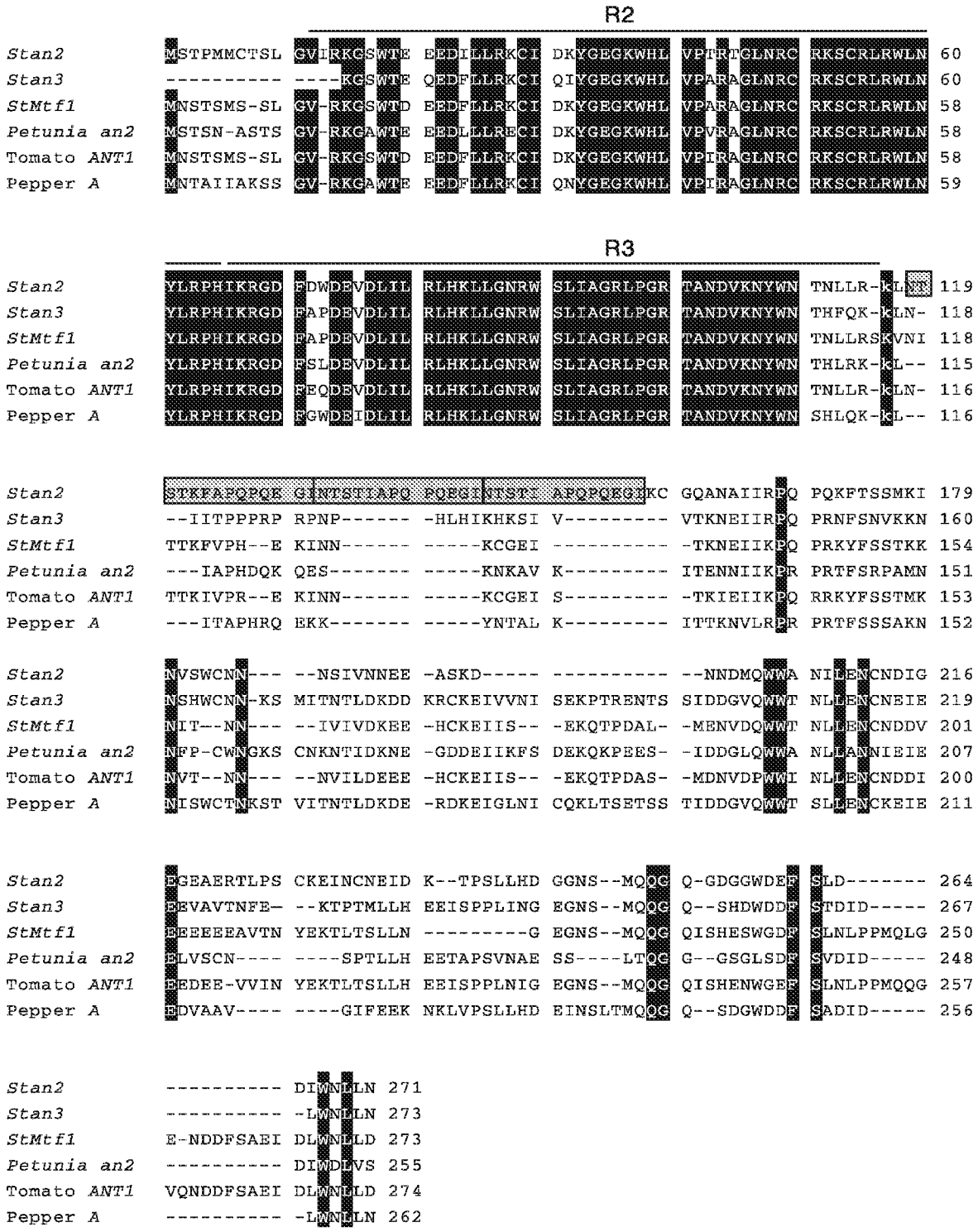


Fig. 4 Alignment of the predicted amino acid sequences of *Stan2* and *Stan3* with those of known regulators of the anthocyanin biosynthetic pathway in the *Solanaceae*. Amino acids conserved across the six peptides—*Stan2*, *Stan3*, potato *StMtf1*, Petunia *an2*, tomato *ANT1*, and pepper *A*—are highlighted in black. The *Stan2*^{816} allele shown contains an almost perfect tandem repeat (highlighted in gray) immediately after the R3 MYB domain: 12 of 14 residues at positions 118–131 are identical to 12 of 13 amino acids at positions 132–144. In addition, *Stan2*^{816} residues 145–157 are a perfect duplication of residues 132–144

expressed in the skin of both red- and white-skinned clones (Fig. 5), as well as in the tuber flesh of all these cultivars (data not shown). Thus, of the two candidate genes, *Stan2* exhibited an expression pattern that was more consistent with the possibility that it corresponds to *D*.

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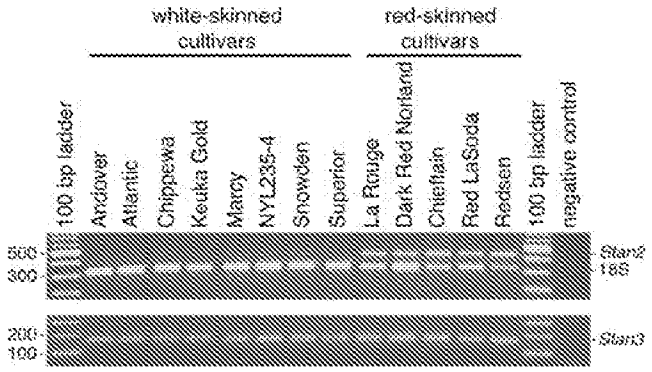


Fig. 5 Expression of *Stan2* and *Stan3* in the periderm of red- and white-skinned potato cultivars. Expression was evaluated by RT-PCR, using mRNA template prepared from the periderm of eight white-skinned and five red-skinned cultivars. For *Stan2* a QuantumRNA™ 18S internal standard was used to control for the success of RT-PCR. The gene names are indicated on the *right side* of each gel. The sizes of selected molecular weight markers, in base pairs, are indicated at *left*. The negative control was an amplification performed without cDNA template

Genomic DNA of both alleles of *Stan2* and *Stan3* from diploid W5281.2 was amplified, cloned, and sequenced. Neither *Stan2* allele from W5281.2 (GenBank accessions AY841130 and AY842506) exhibited the perfect 39 bp/13 amino acid duplication observed in $Stan2^{816}$. One of the W5281.2 alleles, designated $Stan2^{777}$, lacked a *Cla* I restriction site within the second intron (Fig. 6a). Evaluation of 73 colored and 63 white-skinned progeny from a cross between W5281.2 and 07506-01 revealed that $Stan2^{777}$ cosegregated perfectly with colored skin. Results from a subset of progeny are shown in Fig. 6b. One of the two alleles of *Stan3* from W5281.2 (Genbank accession AY842507) also cosegregated perfectly with colored skin in the same 136 progeny (data not shown), demonstrating that *Stan2*, *Stan3*, and *D* are all tightly linked.

Allele-specific expression

Like the structural genes *dfr* and *f3′5′h*, RT-PCR analyses revealed that *Stan2* was expressed in the tuber skin of colored parent W5281.2 and in colored progeny, but not in the tuber skin of white-skinned parent 07506-01 or in white progeny (Fig. 3b). To determine which allele(s) of *Stan2* are expressed in colored progeny, portions of the genomic DNA of both alleles of *Stan2* were also sequenced from 07506-01. Sequence comparison indicated that $Stan2^{777}$ could be differentiated from all other parental alleles by virtue of a polymorphic *Alu* I restriction site in the first exon (Fig. 7a). RT-PCR followed by *Alu* I digestion demonstrated that the only allele expressed in tuber periderm of W5281.2 and colored progeny was $Stan2^{777}$ (Fig. 7b).

Transgenic expression

To test directly whether $Stan2^{777}$ can regulate anthocyanin biosynthesis, the open reading frame of this allele

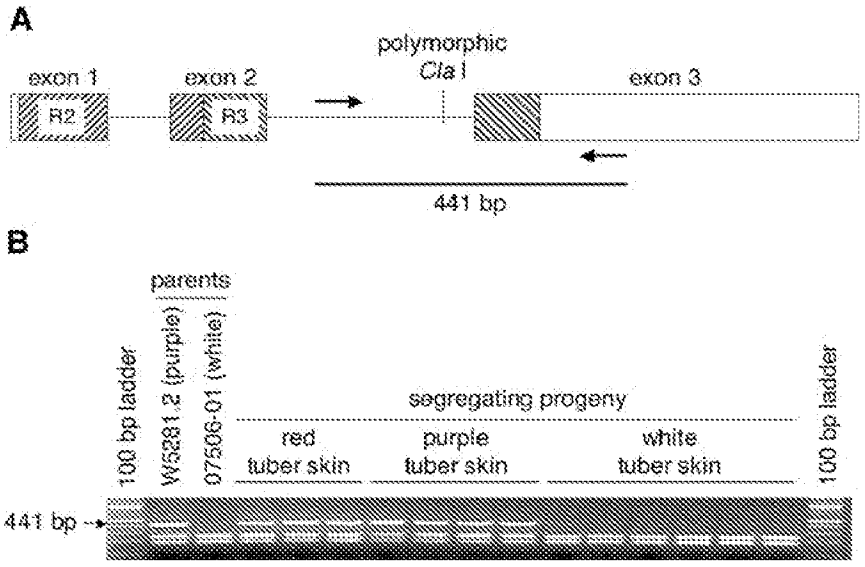


Fig. 6 Structure of *Stan2* and cosegregation of this gene with tuber skin color. **a** The *Stan2* gene contains three exons, indicated with boxes, and two introns, indicated with *horizontal lines*. R2 and R3 MYB DNA-binding domains are indicated with *diagonal hatch lines*. The position of a polymorphic *Cla* I restriction site, which differentiates the two alleles of this gene in W5281.2, is also shown. Arrows indicate binding sites of oligonucleotide primers Stan2-ClaI-F2 and Stan2-ClaI-R1 (see "Materials and Methods"). **b** Primers diagrammed in **a** were used to amplify genomic DNA from parents W5281.2 and 07506-01 as well as from segregating progeny. Products were digested with *Cla* I and then electrophoresed on a 2% agarose gel. The $Stan2^{777}$ allele yields a 441 bp fragment with this assay and was observed to cosegregate with colored tuber skin

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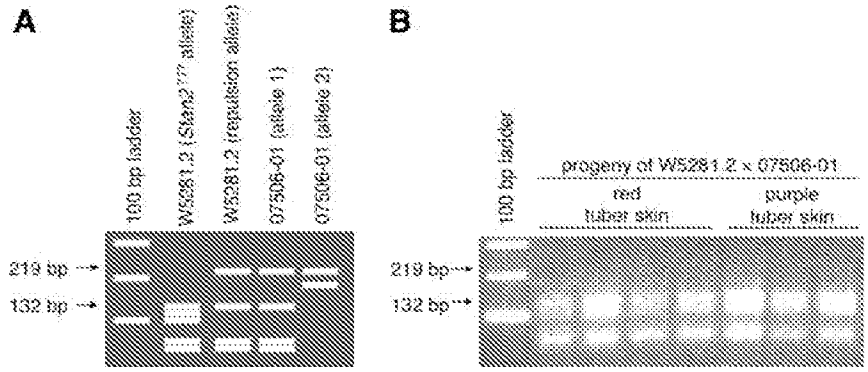


Fig. 7 Only one allele of *Stan2* is expressed in progeny with colored skin. Primers Stan2-F4 and Stan2-ClaI-R1 were used to amplify cDNA prepared from red- and purple-skinned progeny of the cross between W5281.2 and 07506-01. Amplification products were then digested with *Alu* I and separated on a 2% agarose gel to determine which W5281.2 and 07506-01 alleles are transcribed. **a** Predicted fragment sizes. Complementary DNA of *Stan2*^{777} was predicted to give restriction fragments of 132, 119, 100, 52, and 44 bp. Based on genomic DNA sequence, the other allele of W5281.2 and one allele of 07506-01 were predicted to give fragments of 219, 132, 52, and 44 bp, while the remaining allele of 07506-01 was predicted to give fragments of 219 and 186 bp. **b** Actual fragment sizes observed. *Panel* shows an ethidium-bromide-stained gel and demonstrates that only *Stan2*^{777} was expressed

(see "Materials and Methods") was placed into binary vector pPS1, behind a doubled CaMV 35S promoter and tobacco etch translational enhancer, and transformed into the light red-skinned cultivar Désirée. Désirée was chosen for transformation because it is relatively easy to transform and its tuber color demonstrates that it possesses a functional allele at the *R* locus. As the CaMV 35S promoter is known to direct expression in many plant tissues (Sunilkumar et al. 2002), we examined transformed plants to see if anthocyanins accumulated in any tissues that are not normally pink or red in color. Callus of *Stan2*^{777}-transformed Désirée was discernibly more pigmented than that of empty vector-transformed or untransformed Désirée (data not shown). Four independent transformants were selected for further analysis. The youngest foliage of each transformant was reddish-purple in color (Fig. 8a). Tuber skin of the same transgenic lines was deep red in color, while tuber flesh exhibited weak pigmentation in the vascular ring and central pith (Fig. 8a). The foliage of untransformed Désirée, as well as that of empty vector-transformed Désirée, was green (Fig. 8a and data not shown). Control tubers displayed light red skin and white flesh (Fig. 8a and data not shown). The flowers of the four transgenic lines were also dark red in color, in contrast to the faint pink color of untransformed Désirée (data not shown).

Thin layer chromatography showed that although Désirée tubers transformed with *Stan2*^{777} contained more anthocyanin, the anthocyanin profile was qualitatively indistinguishable from that of untransformed Désirée (Fig. 8a). These results demonstrate that *Stan2*^{777} can regulate anthocyanin synthesis and that tissue non-specific overexpression leads to increased anthocyanin accumulation in multiple plant tissues.

The same construct used to transform Désirée was also introduced into the white-skinned cultivar Bintje. Periderm of seven (out of seven) transgenic Bintje lines was purple in color, while tuber flesh exhibited weak pigmentation in the vascular ring (Fig. 8b). Tuber periderm and flesh of Bintje transformed with an empty vector remained white (data not shown). The purple coloration observed in transgenic Bintje suggests that Bintje carries a functional allele at *P*.

For a more direct complementation test, two white-skinned progeny from the cross between W5281.2 and 07506-01 were selected and transformed with the same construct used for Bintje and Désirée. Based on marker and phenotypic analyses (this paper; De Jong et al. 2003a; Jung et al. 2005), one daughter clone was known to have genotype *dd Rr pp* (white skin, white tuber sprouts), while the other daughter clone had genotype *dd Rr Pp* (white skin, purple tuber sprouts). Seven (out of seven) transgenic lines derived from the *dd Rr pp* clone exhibited red coloration in tuber skin and tuber flesh (Fig. 8c), while one (out of three) transgenic line derived from the *dd Rr Pp* clone produced tubers with purple skin and no apparent pigmentation in tuber flesh (Fig. 8d).

Allelic composition in tetraploid cultivars and breeding clones

To assess the allelic diversity of *Stan2* in tetraploid germplasm, a portion of the second intron and third exon was amplified from 60 cultivars and breeding clones. The products were then separated at high resolution on a 5% denaturing acrylamide gel. Products of six different sizes were observed (Fig. 9). The fastest migrating band, approximately 400 bp in size and labeled 'F' in Fig. 9, was found in 14 of 15 red-skinned/white-fleshed cultivars and breeding

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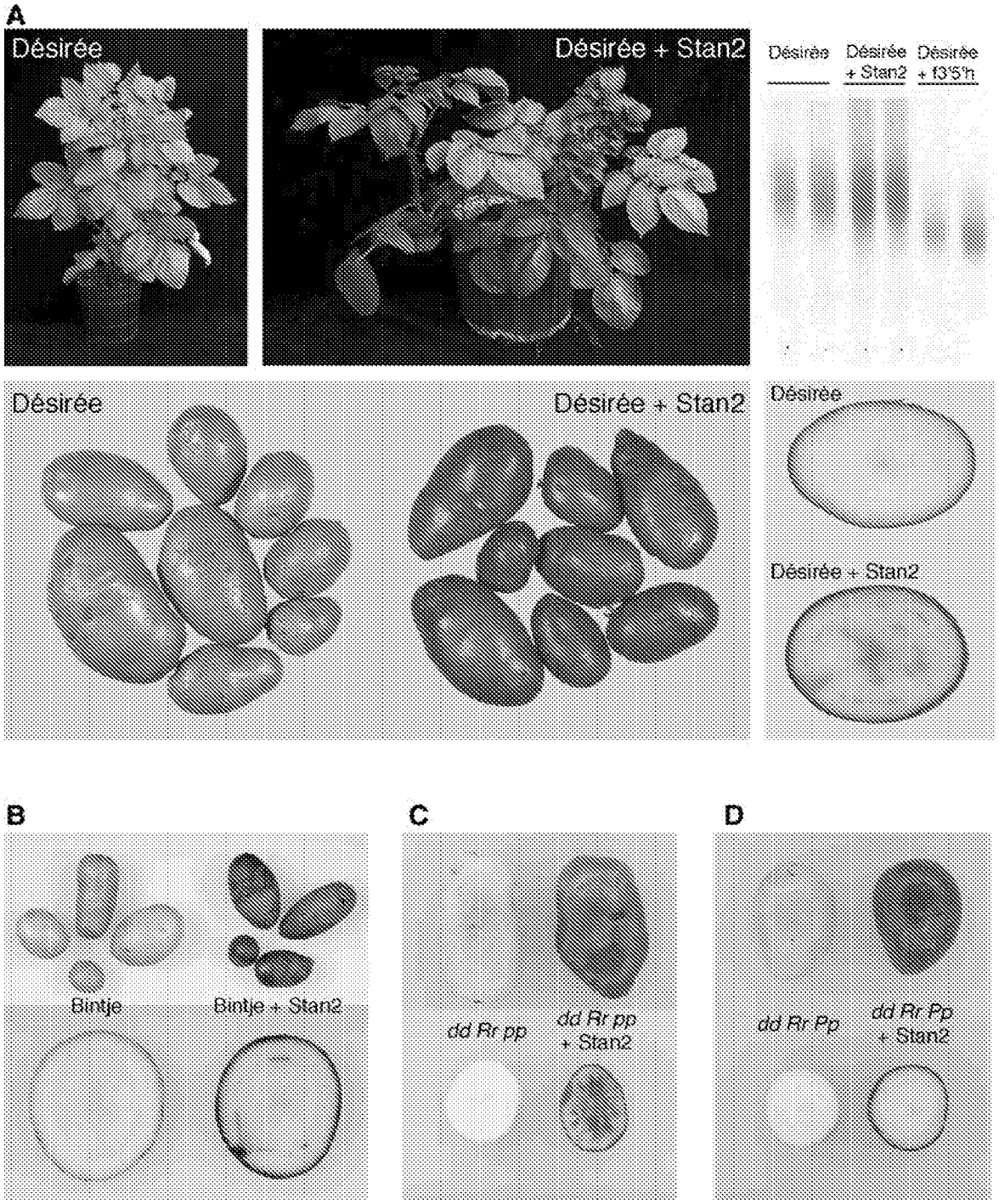


Fig. 8 Transformation of the cultivars Désirée and Bintje, and two progeny from a cross between W5281.2 and 07506-01, with *Stan2*. The open reading frame of the R2R3 MYB domain allele *Stan2*^{777} was placed into the vector pPS1 under the control of a doubled 35S promoter and introduced via *Agrobacterium*-mediated transformation. Tubers were harvested from mature plants and photographed several days later. **a** Foliage, tubers, and tuber flesh of cultivar Désirée, as well as corresponding tissues from a Désirée plant transformed with *Stan2*^{777}. *Top right* thin layer chromatography of anthocyanin pigments isolated from tuber skin of untransformed Désirée, Désirée transformed with *Stan2*^{777}, and Désirée transformed with a functional allele of *f3′5′h* (Jung et al. 2005). Pigment samples were applied near the bottom of the TLC plate shown and developed with a solvent comprised butanol/2 N HCl. The orange color and faster migration of the sample prepared from Désirée and Désirée transformed with *Stan2*^{777} is characteristic of derivatives of pelargonidin, while the purple color and slower migration in the remaining two lanes is characteristic of derivatives of petunidin (Dodds and Long 1955). **b** Tuber and tuber flesh of Bintje, as well as corresponding tissues from Bintje transformed with *Stan2*^{777}. **c** Tuber and tuber flesh of a diploid clone with genotype *dd Rr pp*, as well as corresponding tissues from the same clone transformed with *Stan2*^{777}. **d** Tuber and tuber flesh of a diploid clone with genotype *dd Rr Pp*, as well as corresponding tissues from the same clone transformed with *Stan2*^{777}

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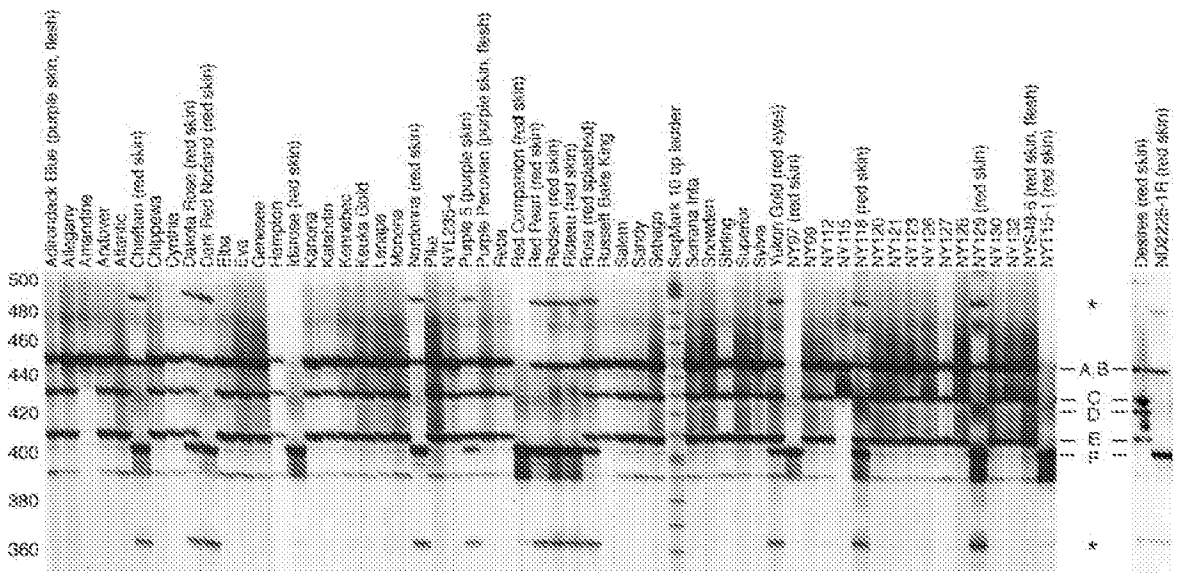


Fig. 9 An allele of *Stan2* common to most red-skinned cultivars but absent in white-skinned clones. A portion of the *Stan2* gene was amplified from 60 tetraploid cultivars and breeding clones with primers Stan2-ClaI-F2 and Stan2-ClaI-R1 and then separated on a 5% denaturing acrylamide gel. Six different fragments, labeled A through F, were observed. Relative to the SeqMark 10 base pair ladder, band A migrated at 447, band B at 445, band C at 430, band D at 423, band E at 408, and band F at 402 bp. The bands marked by asterisks appear to result from recombination during PCR. Amplification of individual plasmids containing genomic DNA corresponding to $Stan2^{777}$ and $Stan2^{816}$ gave rise to bands B and F, respectively, while amplification of a mixture of these two plasmids gave the two expected bands as well as the bands marked with an asterisk (data not shown). Recombination during PCR has been reported by others (Meyerhans et al. 1990; Judo et al. 1998)

clones as well as in 'Purple 5', a purple-skinned/white-fleshed clone. The same band was absent in all 39 white-skinned clones tested. Cloning and sequencing band F from Idarose and NY97 revealed that it corresponded to $Stan2^{816}$. Amplification of a plasmid containing genomic DNA of $Stan2^{777}$ yielded a product that comigrated with band B (data not shown). Although the third exon of $Stan2^{816}$ is 39 bp longer than the third exon of $Stan2^{777}$, it nevertheless gives rise to a smaller PCR product with this assay because the second intron of $Stan2^{816}$ (genomic DNA sequence deposited as accession AY841128) is 82 nucleotides shorter than that of $Stan2^{777}$. Band F was also observed in one cultivar with red tuber eyes (Yukon Gold) and one cultivar (Rosa) with a red 'splashed' phenotype (red eyes and areas of red skin around the eyes). The only red-skinned/white-fleshed cultivar tested that did not contain $Stan2^{816}$ was Désirée. Désirée was also unique in displaying a fragment (labeled 'D' in Fig. 9) that was absent in all 59 other clones.

Three potato clones with pigmented skin and pigmented flesh (Adirondack Blue, Purple Peruvian, NYS48-6) also did not contain $Stan2^{816}$ (Fig. 9).

Bands C and E were either both present or both absent in all clones tested, implying that the underlying alleles are tightly linked, possibly as (part of) a tandem array. Band E was 39 nucleotides shorter than band A, suggesting that the alleles underlying bands A and E differ in copy number of the same 39 bp region that is duplicated in $Stan2^{816}$.

Discussion

D is required for the accumulation of anthocyanin pigments in tuber skin. Several genes known to control tissue-specific accumulation of anthocyanins in model systems are transcription factors that regulate the expression of multiple anthocyanin biosynthetic genes, e.g., the MYB domain gene *an2* of *Petunia* (Quattrocchio et al. 1999) and the basic helix-loop-helix gene *R* of maize (Consonni et al. 1997). To test the hypothesis that *D* might also act to regulate the activity of multiple genes, the expression of three unlinked structural genes (*f3h*, *dfr*, and *f3′5′h*) were evaluated in a potato population segregating for pigmented skin. All three were expressed in progeny with colored tuber skin, while *dfr* and *f3′5′h* were not expressed, and *f3h* was only weakly expressed, in progeny with white tuber skin. Two candidates for *D* (*Stan2* and *Stan3*) were isolated from a potato cDNA library prepared from pigmented tuber skin, using *Petunia an2* as a hybridization probe. Both candidates cosegregated with *D*, but only *Stan2* was expressed specifically in potatoes with colored skin. When placed under the control of a doubled 35S promoter and transformed into the light red-skinned variety Désirée, the white-skinned variety Bintje, and two white-skinned diploids, *Stan2* directed increased accumulation of anthocyanins in tuber skin, as well as in foliage. These results collectively support the hypothesis that *Stan2* corresponds to *D*. The difference between dominant and recessive alleles

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of *D* appears to be at the level of transcription, as functional alleles were transcribed while non-functional alleles were not. We did not observe any premature stop codons or splice-site alterations in the non-expressed alleles sequenced.

In petunia flowers the structural gene *f3h* is expressed independently from anthocyanin regulatory genes (Quattrocchio et al. 1993, 1998), while in potato tubers *f3h* appears to be regulated, at least in part, by the *D* locus (Fig. 4). The differential regulation of *f3h* between these two closely related species, albeit in different tissues, is interesting. Lewis et al. (1998) observed a small but significant difference ($p < 0.05$) in flavonoid levels (other than anthocyanins) between purple/red-skinned and white-skinned tuber classes, with colored skin containing higher concentrations of flavonoids than white skin. Increased expression of *f3h* in colored potato skin may be responsible for this difference, although further study is needed to test this relationship more rigorously.

The function of *Stan3* remains unknown at present. It may be a pseudogene, as we were unable to obtain a full-length cDNA, or it may regulate a function unrelated to anthocyanin production. Testing the function of individual members of closely related gene families has always been challenging, although the artificial miRNA approach outlined by Schwab et al. (2006) and Warthmann et al. (2008) may provide a way to do so.

In tetraploid potato, additional gene symbols have been assigned to loci that direct anthocyanin expression in tuber periderm (*E*) (Lunden 1937, 1960) or tuber phelloderm (the *R* locus of Lunden 1960, 1974). We consider it most likely that Lunden's *E* and *R* are simply alleles of *D*, although further experimentation will nevertheless be required to confirm/disprove this. Another locus that has been described at the tetraploid level is *M*. When present together with *E*, *M* has been reported to restrict tuber pigment to the areas around the tuber eyes (Howard 1970), which could explain why the cultivars Yukon Gold and Rosa both appear to possess a functional allele of *D* (Fig. 9), yet have pigment restricted to areas around the tuber eye.

It has recently been reported that application of the rare-earth metal Cerium (Ce [IV]) to potato cell tissue cultures induces many anthocyanin structural genes (*chs*, *f3h*, *f3′5′h*, *dfr* and *3gt*) and increases anthocyanin production (Qineng et al. 2006). Since these parallel the phenotypes mediated by *D*, Cerium may exert its effects by activating expression of *D* or a closely related gene, or by substituting for R2R3 MYB gene function.

The completely sequenced genome of *Arabidopsis thaliana* codes for approximately 126 R2R3 MYB domain genes (Stracke et al. 2001; Yanhui et al. 2006). Two of these, AtMYB75 (also known as *Pap*1) (Kranz et al. 1998; Romero et al. 1998; Borevitz et al. 2000) and AtMYB90 (*Pap*2) (Kranz et al. 1998; Borevitz et al. 2000), are known to regulate anthocyanin synthesis. These two genes, as well as two other R2R3 MYB genes, AtMYB113 and AtMYB114, are closely related in sequence (Stracke et al. 2001). Three of these genes—AtMYB113, AtMYB114, and AtMYB90—are tightly linked in a tandem array on chromosome 1, while AtMYB75 is located approximately 3.5 Mbp away on the same chromosome. The cosegregation of bands C and E in Fig. 9 suggests that at least one haplotype of the *D* locus contains a tandem array of two or more R2R3 MYB domain genes. Previous studies have shown that potato loci conditioning tissue-specific accumulation of anthocyanins are tightly linked to each other and to *D*. These include *B* (where a hierarchy of alleles is required for pigmentation of seed spot, nodal band, floral abscission layer, and tuber eyebrow), *F* (required for anthocyanin expression in flowers), *Ow* (pigmentation of ovary wall), *Pf* (pigmentation of tuber flesh), *Pw* (pigmentation of leaf whorl), *Ul* (pigmentation of the underside of leaves), *Pd* (pigmentation of the dorsal side of leaves), and *Pv* (pigmentation of the ventral side of leaves) (Kessel and Rowe 1974; De Jong 1987, 1991; Dodds and Long 1956; De Jong and Rowe 1972; Garg et al. 1981). One or more of these classical loci may correspond to bands C and/or E, or other bands, of Fig. 9.

Several genes or quantitative trait loci that influence tissue-specific anthocyanin expression in other Solanceous species—such as pepper *A*, tomato *ag*, and eggplant *fap* 10.1—map to regions comparable to *D* in their respective genomes (reviewed in De Jong et al. 2004), suggesting that all code for similar MYB domain genes. Indeed evidence was recently presented that pepper *A*, which conditions anthocyanin expression in the foliage, flower, and immature fruit, encodes a gene related to *an2* (Borovsky et al. 2004). The precedents of *an2* and *A*, and now *D*, strongly suggest that evolution and subsequent selection for novel tissue-specific expression patterns of R2R3 MYB domain genes in the *Solanaceae* is responsible for much of the anthocyanin-mediated, visually conspicuous phenotypic variation that is so prevalent in this economically important and diverse plant family.

Evaluation of tetraploid germplasm with a simple PCR assay revealed that the $Stan2^{816}$ allele was common to 14/15 red-skinned cultivars and absent in all white-skinned cultivars tested. The unique size of this allele should make it simple to track in potato breeding programs, e.g., for the purpose of developing homozygous (quadruplex) parental lines. In maize, the expression level of transcriptional regulators of anthocyanin biosynthesis is directly correlated with the amount of anthocyanin produced (Patterson et al. 1993; Hollick et al. 2000). Consistent with this, overexpression of *Stan2* in transgenic Désirée increased the amount of anthocyanin in tuber skin. De Jong et al. (2003b) and

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Salaman (1910) have previously postulated that dosage of functional alleles at *D* correlates with the intensity of red tuber color. Figure 9 suggests, but does not prove, that four clones—IdaRose, Red Companion, NY97 and NYT15-1—are quadruplex for the 816 allele, as the PCR assay only amplified band F from each of them. It is thus of interest to note that NY97 and NYT15-1 are two of the deepest red-skinned clones ever developed by the Cornell University breeding program. With the availability of *Stan2*^{816} sequence it should now be possible to test whether dosage of this allele, or other functional alleles, influence intensity of tuber color.

We have previously presented evidence that a functional allele at *R*, also required for red tuber color, is present in many white-skinned potatoes (De Jong et al. 2003a). In the current study, we found no white potatoes that possess *Stan2*^{816}. Taken together these observations suggest that white-skinned cultivars are most frequently white because they lack a functional allele of *D*, not because they lack a functional allele of *R* (or *P*).

One curious result in the present study was that the flesh of transgenic tubers of Désirée, Bintje, and two progeny from the cross between W5281.2 and 07506-01 was not completely red or purple. In diploids, pigmented flesh is mediated by *Pf*, a locus tightly linked to *D* (De Jong 1987). The pigmented flesh trait exhibits variable expressivity, i.e., it is not unusual in a single segregating cross to observe some progeny with completely colored flesh, some progeny with only traces of pigmentation, and other progeny with a wide range of intermediate phenotypes. Désirée, Bintje and the two transformed diploid clones may have a genetic background that limits the extent to which flesh can be pigmented. Alternatively the 35S promoter used to drive *Stan2* expression may not function well in all tuber tissues. Sung et al. (1994) reported that GUS marker gene expression in mature tubers from a plant transformed with a 35S:GUS construct was higher in tuber vasculature than in other tuber tissues, although GUS was expressed in most tissues from young tubers. Rommens et al. (2008) recently transformed Bintje with another *Stan2*-like MYB gene, *StMtf1*, and observed similar, variable tuber flesh coloration, when the gene was expressed under the control of two alternative constitutive promoters (ubiquitin-7 and GBSS). The combined results from the current and Rommens et al. (2008) studies suggest that, at least in Bintje, MYB gene expression is not sufficient for complete coloration of tuber flesh. Quantitative trait loci that influence the degree of tuber flesh pigmentation have recently been mapped on chromosomes 5, 8, and 9 (Zhang et al. 2009a); allelic configurations at these, and/or other unknown loci, may influence the tuber flesh phenotype when *Stan2* is constitutively expressed.

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Mapping of intra-locus duplications and introgressed DNA: aids to map-based cloning of genes from complex genomes illustrated by physical analysis of the *Rx* locus in tetraploid potato

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Abstract We describe here novel approaches to high-resolution mapping of repeated and introgressed DNA which may prove generally useful in map-based cloning from complex genomes. These approaches were developed in order to clone the *Rx* locus in potato. First, we prepared a BAC library from a tetraploid plant carrying *Rx* in the duplex condition (*Rx*, *Rx*, *rx*, *rx*). BAC clones were then isolated with close markers on either side of *Rx*. However these clones did not extend across *Rx*: in the cloned DNA the closest markers to *Rx* were separated by single recombination events on either side of *Rx*. To bridge the gap we exploited the finding that the BAC clones on the right side of *Rx* contained resistance-gene homologues. Anticipating that there would be duplicated copies of these resistance gene homologues in the vicinity of *Rx*, we used low-stringency PCR conditions to identify additional markers. One of these markers was completely linked to *Rx* in our mapping population and was used to isolate a BAC (BAC77) that had not been previously identified by screening with *Rx*-flanking markers. Based on two criteria it was concluded that BAC77 spans *Rx*. There was a chromosomal recombination in one plant of our mapping population that separated the BAC77 right end from *Rx*. On the other side of *Rx* it was found that the BAC77 left end was outside the region of DNA carrying *Rx* that had been introgressed into potato cv Amaryl from a *Solanum tuberosum* ssp. *andigena* accession CPC1673.

Key words Map-based cloning · BAC · *Rx* · PVX · Potato

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Introduction

We are currently adopting a map-based cloning approach to the molecular cloning of the *Rx* locus in potato (Cockerham 1970). *Rx* confers extreme resistance against potato virus X (PVX) and, although PVX is not a major problem in potato production, there are several reasons for the cloning and further analysis of this gene. The genetical analysis of the *Rx*-mediated resistance in both the host plant and the pathogen shows that it fits the gene-for-gene paradigm (Flor 1971; Keen 1990), indicating that the findings from *Rx*-mediated resistance will be relevant to resistance against fungi, bacteria and other pathogens. Furthermore, analysis of *Rx*-mediated resistance in protoplasts prompted the suggestion that it may be possible to produce broad spectrum virus resistance by the manipulation of *Rx*-mediated resistance. This protoplast analysis demonstrated that the specificity of *Rx*-mediated resistance is determined at the level of recognition and that there is a response phase that can suppress several viruses, and not only PVX (Köhm et al. 1993; Bendahmane et al. 1995).

Until now, no genes have been cloned from potato using map-based approaches despite the high-resolution mapping of several loci in the potato genome (Ballvora et al. 1995; Meksem et al. 1995; Brigneti et al.

1997; De Jong et al. 1997). In fact, only in *Arabidopsis thaliana* have map-based approaches been widely applied (Giraudat et al. 1992; Bent et al. 1994; Mindrinos et al. 1994; Busch et al. 1996; Lukowitz et al. 1996; Ryals et al. 1997). In other plants there are only few reports of genes that have been cloned by a map-based approach (Martin et al. 1993; Dixon et al. 1996; Büschges et al. 1997). In the present paper we describe an extension of our previous mapping of *Rx* that was designed to explore the feasibility of a map-based cloning approach. From our findings we developed novel strategies leading to identification of a bacterial artificial chromosome (BAC; Shizuya et al. 1992) clone that spans *Rx*. This approach was successful despite the suppression of recombination and the depletion of AFLP (Vos et al. 1995) and RFLP markers in the vicinity of *Rx*, and could be applied to other map-based cloning projects in plants with complex genomes.

Materials and methods

Plant material

F_1 seeds from the cross between potato cv Cara (*Rx*, *rx*, *rx*, *rx*) and cv Huinkel (*rx*, *rx*, *rx*, *rx*) were obtained from the Plant Breeding Institute, Cambridge. The progeny of this cross (370 individuals) was used in initial experiments (Bendahmane et al. 1997) to identify the chromosomal position of *Rx*, and to assign RFLP markers linked to *Rx*. The self pollinated (S_1) progeny of cv Cara (1350 plants) (Bendahmane et al. 1997) was used as a mapping population to position *Rx* with respect to RFLP, AFLP and BAC-derived PCR markers. An additional 1438 plants of S1 cv Cara were employed to ascertain whether BAC77 spans *Rx*. A diploid potato clone SH83-92-488 (Rouppe van der Voort et al. 1997) having cv Amaryl (*RxGpa2*, *rxgpa2*) in its pedigree, cv Amaryl and self-pollinated offspring of *Solanum tuberosum* ssp. *andigena* accession CPC1673 (encoded CPC3520 and obtained from the Commonwealth Potato Collection, Scottish Crop Research Institute, Dundee, UK) were used for 'introgression mapping'.

Construction of the potato BAC library

High-molecular-weight DNA was prepared in agarose plugs from potato protoplasts essentially as described in Bendahmane et al. (1997). The agarose plugs were dialysed three times for 30 min against TE buffer (10 mM Tris-HCl pH 7.5, 1 mM EDTA), once at room temperature and twice at 4°C. The plugs were then equilibrated in *Hin*dIII buffer (10 mM Tris-HCl, 10 mM $MgCl_2$, 50 mM NaCl, 1 mM DTT, pH 7.9) twice on ice for 1 h. Half of each plug (about 5 µg of DNA) was transferred to a test tube containing 360 µl of *Hin*dIII buffer and 10–15 units of *Hin*dIII restriction enzyme. The enzyme was allowed to diffuse into a plug at 4°C for 1 h and the digestion was carried out at 37°C for 30 min. The reaction was stopped by adding 1 ml of 0.5 M EDTA. Plugs were washed in 0.5 × TBE (45 mM Tris-borate pH 8.0, 1 mM EDTA), loaded into a 1% low-melting-point agarose gel and subjected to contour-clamped homogeneous electric field (CHEF; Chu, 1989) electrophoresis in a CHEF DR II system (Bio-Rad Laboratories, USA) in 0.5 × TBE buffer at 150 V for 10 h at 4°C and a constant pulse time of 5 s or 8 s. Compression zones containing the DNA fragments of approximately 100 kb or approximately 150 kb were excised from the gel and dialysed against 30 ml of TE in a 15-cm Petri dish for 2 h at 4°C. Dialysed agarose slices then were transferred to a 1.5 ml test tube, melted at 65°C for 10 min, and digested with 1 unit of *Gelase* (Epicentre Technologies, USA) per 100 µg of an agarose gel for 1 h at 45°C.

The size-selected potato DNA (25–50 ng) was ligated to 25–50 ng of *Hin*dIII-digested and de-phosphorylated pBeloBAC11 vector, kindly provided by Dr. H. Shizuya (University of Southern California, Los Angeles; Shizuya et al. 1992), using 400–800 units of T4 DNA Ligase (New England BioLabs, USA) at 16°C for 24 h in a total volume of 50 µl. The ligation products were dialysed against 1 × TE using 0.025 µM MF-Millipore membrane filter (Millipore, UK) at 4°C for 2 h and 30 min at room temperature using the 'drop dialysis' method of Maruzyk and Sergeant (1980).

Transformation of *Escherichia coli* DH10B cells was carried out by electroporation using a BRL Cell-Popagator System (Life Technologies Ltd, UK). To 20 µl of electro-competent cells, 0.5–3 µl of ligation mixture was added. After electroporation, *E. coli* cells were mixed with 1 ml of SOC medium (0.5% yeast extract, 2% tryptone, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose) and incubated at 37°C for 1 h with gentle shaking (80 rpm). The cells were spread on Luria broth (LB) agar plates containing chloramphenicol (12.5 µg/ml), 5-bromo-4-chloro-3-indolyl-β-D-galactoside (Xgal) (40 µg/ml) and isopropyl-1-thio-β-D-galactoside (IPTG) (0.12 mg/ml). Plates were incubated at 37°C for 24 h.

DNA from the compression zones of 100 and 150 kb led to clones with an average insert size of 100 kb. The 100-kb DNA produced approximately 1000 white colonies per µl of ligation mixture, whereas the 150-kb DNA produced only 150 white colonies per µl of ligation mixture. Approximately 92 000 white colonies were picked individually into 384-well microtiter plates (Genetix, UK) containing LB freezing buffer [36 mM K_2HPO_4, 13.2 mM KH_2PO_4, 1.7 mM citrate, 0.4 mM $MgSO_4$, 6.8 mM $(NH_4)_2SO_4$, 4.4% V/V glycerol, 12.5 µg/ml of chloramphenicol in LB medium], grown overnight at 37°C and stored at −80°C. We also prepared 100 bacterial pools containing about 500–1000 white colonies each (these pools also contained approximately 500–1500 blue bacterial colonies with an empty pBeloBAC11) by scraping the colonies from agar plates into the LB medium containing 18% glycerol and 12.5 µg/ml chloramphenicol using a sterile glass spreader. These pools were also stored at −80°C.

Screening of the potato BAC library

The potato BAC library was screened with IPM3, IPM4 and IPM5 CAPS markers, corresponding to the AFLP markers PM3, PM4 and PM5 flanking the *Rx* locus (Bendahmane et al. 1997), as follows.

For the first part of the library of 92 160 clones stored in 384-well microtiter plates the plasmid DNA was isolated using the standard alkaline-lysis protocol (Heilig et al. 1997) from pooled bacteria of each plate to produce 240 plate pools. Aliquots of these plate pools were combined to prepare 26 'superpools' each of which contains DNA from nine plate pools, and one superpool containing DNA from six plate pools. To identify individual BAC clones carrying the CAPS markers, the superpools and then the corresponding plate pools were screened. Once an individual plate had been identified the clones corresponding to each of the 24 columns of the positive plate were grown for 3–4 h at 37°C in LB medium and PCR was carried out on 3 µl of bacteria. After identification of a positive column a colony PCR on each of the corresponding 16 colonies of this column was carried out leading to the identification of a single positive BAC clone.

The second part of the library was stored as 100 pools of approximately 1000 clones. The plasmid DNA was isolated from each pool of clones using a standard alkaline-lysis protocol and PCR was carried out to identify the positive pool. The bacteria corresponding to the positive pool were diluted, plated on LB agar plates and then

colony hybridisation was carried out as described in Sambrook et al. (1989) using ^{32}P-labelled DNA probes corresponding to CAPS markers. PCR with the corresponding CAPS primers was used to distinguish hybridising colonies carrying the markers that had been previously mapped from the homologues located elsewhere in the genome.

Analysis of the BAC library

BACs containing potato DNA were isolated from 5-ml overnight cultures (LB medium supplemented with 12.5 mg/ml of chloramphenicol) using the standard alkaline-lysis miniprep protocol (Engebrecht et al. 1997) and re-suspended in 50 µl of TE. Plasmid DNA (10 µl) was digested with *Not*I for 3 h at 37°C to release the insert DNA from the pBeloBAC11 vector. The digested DNA was separated by CHEF electrophoresis in a 1% agarose gel in 0.5 × TBE at 4°C using a BIO-RAD CHEF DR II system (Bio-Rad Laboratories, USA) at 150 V with a constant pulse time of 14 s for 16 h.

Isolation and characterization of BAC ends

An inverse polymerase chain reaction (IPCR; Ochman et al. 1990) was used to isolate the right and left end-sequences of insert DNAs. BAC DNA was isolated and digested separately with *Nla*III, *Hpa*II, *Mse*I, *Hin*P1I, *Pvu*II, and *Hae*III (for isolation of a left end-sequence) or with *Rsa*I, *Sac*I, *Eco*RI, *Hae*III, *Mae*II, *Mse*I, *Pvu*II, and *Hin*P1I (for isolation of a right end-sequence) for 4 h at 37°C and re-circularised by self-ligation for 2 h at 20°C. Ligations were carried out using 5–50 ng of digested DNA and 5–10 units of

Table 1 PCR-based markers linked to the *Rx* locus

Marker	Primers	PCR conditions[b]	Restriction enzyme
117L	5′-CCTAGCGTAGAGCGGTGTATCCA 5′-GTAGACATTTAATAATTCGTCGATAC	94°C, 15 s × 35 cycles 57°C, 20 s × 35 cycles 72°C, 120 s × 35 cycles	*Rsa*I
191L	5′-ACAAATTGTATAATTATAGTGATACG 5′-CAAGACATTAATTAACCAAACAATGG	94°C, 15 s × 35 cycles 50°C, 15 s × 35 cycles 72°C, 120 s × 35 cycles	*Eco*RI
77L	5′-GCTTCTAAACTCTAAATTCAGATTC 5′-CATGTGCGGACTCGTTCTTTTGTAG	94°C, 15 s × 35 cycles 64°C, 15 s × 35 cycles 72°C, 60 s × 35 cycles	*Alu*I
IPM4	5′-GTACTGGAGAGCTAGTAGTGATCA 5′-GAACACCTTAACTACACGCTGCAGG	94°C, 15 s × 35 cycles 62°C, 15 s × 35 cycles 72°C, 90 s × 35 cycles	*Taq*I
77R[a]	5′-GAAAGACAATTCCAGTGTGATGCG 5′-CAGGTAAGCCTCCTCATAACATGC	94°C, 15 s × 35 cycles 66°C, 15 s × 35 cycles 72°C, 60 s × 35 cycles	
45L[a]	5′-GGAGTCAATGCAGGGTCTATGGAA 5′-CTCATTTGACACTTCTCGAACACA	94°C, 15 s × 35 cycles 62°C, 15 s × 35 cycles 72°C, 50 s × 35 cycles	
221R[a]	5′-GCTTACATTTGCTCGAAGAAGCCAC 5′-CCTTAATAATCAATAGATTCAACTCG	94°C, 15 s × 35 cycles 60°C, 15 s × 35 cycles 72°C, 60 s × 35 cycles	
111L[a]	5′-CCGAGTTTGCTCGATTCCGAGTTTT 5′-CTAAGGGATCCACTAGTCTAATTTG	94°C, 15 s × 35 cycles 62°C, 15 s × 35 cycles 72°C, 60 s × 35 cycles	
111R[a]	5′-CCACTGTGTAAGGGTCAACTATAGTC 5′-GAGATGAAGATTTTCTTGTCTGATGG	94°C, 15 s × 35 cycles 65°C, 15 s × 35 cycles 72°C, 90 s × 35 cycles	
73L	5′-CATTTCCTGAATTGCTTCCGACTTC 5′-CCATGAAAATTGTTATCACTGAGGTC	94°C, 15 s × 35 cycles 60°C, 15 s × 35 cycles 72°C, 50 s × 35 cycles	*Alu*I
218R	5′-GATTACAGTTGTGAATTAGTTCGGTA 5′-GCAACAGATATATTCCACTTACCATTC	94°C, 15 s × 35 cycles 60°C, 15 s × 35 cycles 72°C, 90 s × 35 cycles	*Alu*I
218L[a]	5′-CTTAACAAACCTATCATATTGGCCAT 5′-AGCTTCACATTGAACCAGAGGCCT	94°C, 15 s × 35 cycles 62°C, 15 s × 35 cycles 72°C, 90 s × 35 cycles	

[a] These markers are allele-specific (i.e. marker primers are able to amplify only an allele linked in *cis* to *Rx*). All other markers were CAPS revealed in the *Rx*-linked form by digestion with a corresponding enzyme (right hand column)

[b] The PCR conditions are the standard PCR conditions used with these primers. Some of the primers were also used in low-stringency PCR with a primer annealing temperature at 10°C below the temperature shown

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T4 DNA Ligase (Boehringer Mannheim, Germany) in a total volume of 100 µl. PCR amplification of the re-circularised DNA was carried out using 3 µl of self-ligated DNA as the template. AB1 (5′-CCTAAATAGCTTGGCGTAATCATG-3′) and AB2 (5′-TGACACTATAGAATACTCAAGCTT-3′) primers were used for PCR-amplification of the left end-sequence of insert DNA, AB3 (5′-CGACCTGCAGGCATGCAAGCTT-3′), while AB4 (5′-ACTCTAGAGGATCCCCGGGTAC-3′) primers were used for PCR-amplification of the right end-sequence of insert DNA. PCR conditions were as follows: 94°C for 15 s, 60°C for 15 s, 72°C for 90 s – for 35 cycles. PCR products were digested simultaneously with *Hin*dIII and the restriction enzyme used in the preparation of the IPCR DNA template. The released insert ends were gel-purified and cloned into pGEM-3Z(f+) (Promega, USA). Sequences of the clones containing approximately 1–2 kb inserts were determined using a 377 or 373 DNA Sequencing System (Applied Biosystems, UK), and these sequences were employed to design PCR primers for amplification of the BAC end-region in potato genomic DNA.

The sequences of primers and the PCR conditions used in the amplification of each BAC end-sequence are summarised in Table 1. The BAC end-sequences were named according to the name of the BAC, followed by the letter L for the left end- or by the letter R for the right end-sequence. For example, the left and the right end-sequences of BAC218 are 218L and 218R, respectively.

The translated versions of BAC end-sequences and of other markers in the vicinity of *Rx* were compared with the PDB, SWISS-PROT, PIR(R), GenPept, and GenPept databases using BLAST (Altschul et al. 1990).

Results

Genome representation in a BAC library

Our previous analysis of *Rx* in potato was based on potato cultivar (cv) Cara carrying *Rx* in a simplex condition (*Rx*, *rx*, *rx*, *rx*). To increase the probability of cloning *Rx*, we prepared a BAC library of 160 000 clones from plant SC-781 which is a self-pollinated progeny of cv Cara carrying *Rx* in the duplex condition (*Rx*, *Rx*, *rx*, *rx*). Based on the average insert size of 100 kb in the BAC clones (data not shown), a haploid genome size of 900 000 kb in potato (Arumuganathan and Early 1991), and taking into account the presence of *Rx* in the duplex condition, we estimate that there is more than a 99% probability that this library carries the DNA of the *Rx* (or indeed any other) locus. This estimate is based on the formula: $N = Ln(1 - P)/Ln(1 - I/G)$, where I is the size of the average cloned fragment in base pairs, and G is the size of the genome in base pairs (Clark and Carbon 1976). According to this formula, for any gene carried in the duplex condition, there would need to be more than 80 000 BAC clones of 100 kb to have a 99% probability of representation in a library.

To confirm the genome representation in this potato BAC library we screened for clones containing single-copy *c*leaved *a*mplified *p*olymorphic *s*equence (CAPS; Konieczny and Ausubel 1993) markers (GP34, PM3, CT99 and CT129) that are linked to *Rx* (Bendahmane et al. 1997). In each instance we obtained between one and eight positive clones (data not shown). At least one clone identified with each marker tested contained an allele linked in *cis* to *Rx*. Therefore, the BAC library is representative and could be used to identify BAC clones spanning many regions of interest in the potato genome.

Screening of the potato BAC library with markers closely linked to and flanking the *Rx* locus

The CAPS markers IPM3 and IPM4 flank *Rx* and span an interval of 0.23 centiMorgans (cM) (Bendahmane et al. 1997). As the estimated physical distance between these markers is only about 100 kb (Bendahmane et al. 1997) we expected to isolate a single BAC clone or a set of clones which span the *Rx* locus.

Using the IPM3 marker to screen the BAC library we identified three BAC clones, BAC167, BAC191 and BAC117, with potato DNA inserts ranging from 100 to 120 kb (Fig. 1). *Dde*I digestion of the IPM3 DNA in these BACs and in potato DNA samples revealed that BAC117 carried the IPM3 allele that was linked in *cis* to *Rx*. The other two BACs, BAC167 and BAC191, contained alleles from a corresponding region of the *rx* chromosomes (Fig. 2a).

To identify the relative genome positions of these BACs we designed pairs of PCR primers based on the sequence of the right and left ends of the insert (see Materials and methods). PCR tests using the BAC DNAs as templates showed that these BACs overlapped in the order BAC167, BAC117, BAC191, *Rx*. The 191L marker was separated from *Rx* by only a single chromosomal recombination event (in plant # 1146) in a mapping population of 1720 plants. In the same

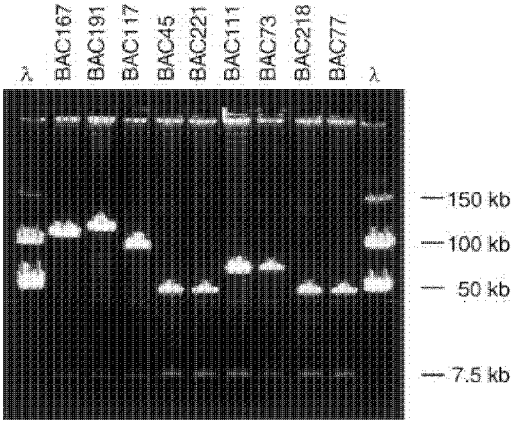


Fig. 1 CHEF electrophoresis analysis of BAC clones containing DNA from *Rx* locus BAC DNA was isolated, digested with *Not*I, and subjected to CHEF electrophoresis as described in Materials and methods. *Names* of the BAC clones are indicated at the top of each line. The high-molecular-weight *Not*I restriction fragments are potato BAC DNA inserts. The DNA fragment of about 7.5 kb present in all BAC lanes corresponds to a linear pBeloBAC11 vector DNA. Concatemers of bacteriophage lambda DNA, 'λ', were used as size standards as indicated on the right side of the panel

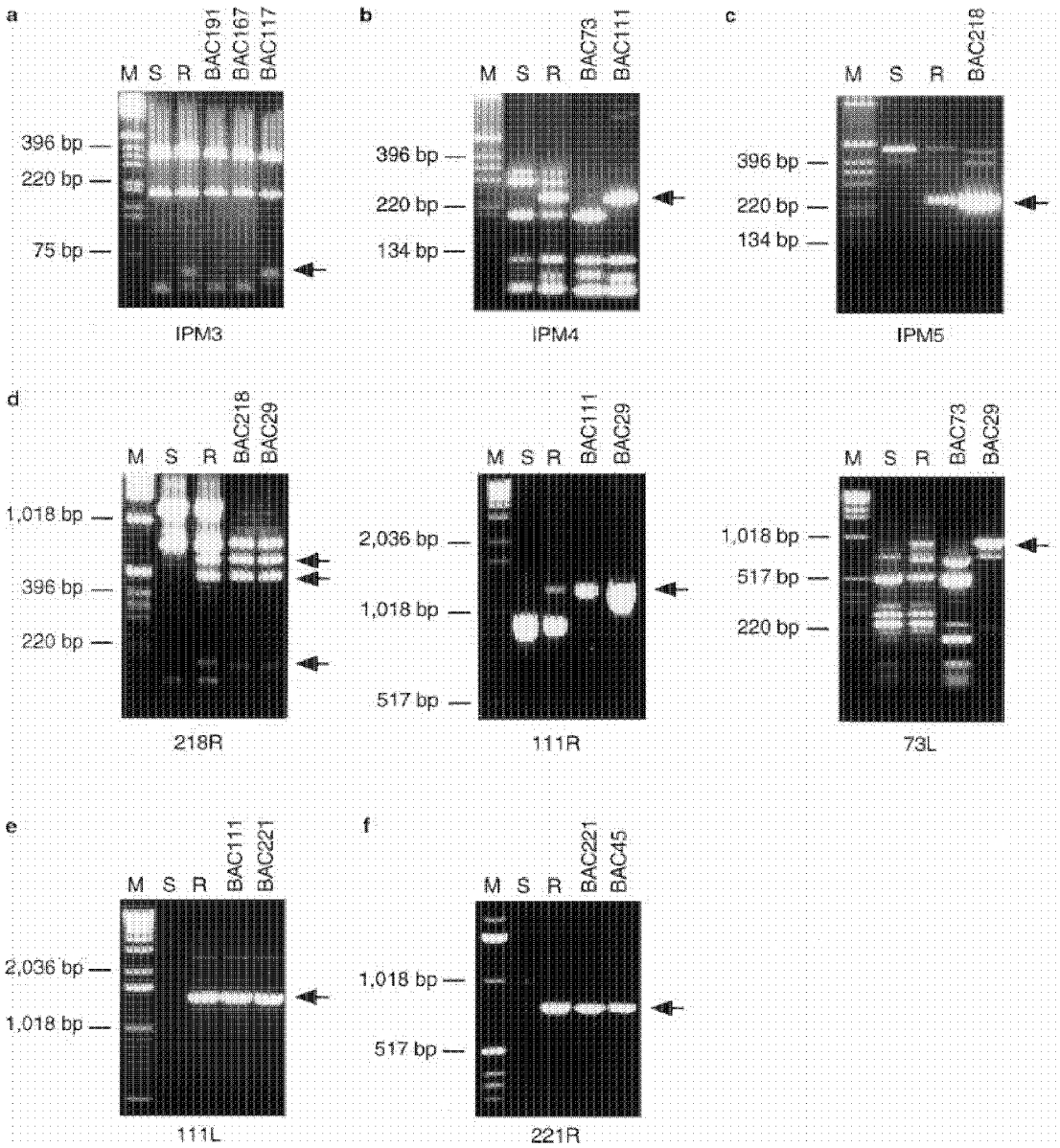


Fig. 2a–f PCR analysis of potato BAC clones identified with markers from the *Rx* locus Pooled DNA of 20 *Rx* plants (*R*), pooled DNA of 20 *rx* plants (*S*), and DNA of BAC clones was subjected to PCR using the primers indicated at the bottom of each panel. *Names* of the BAC clones are indicated at the top of each lane. PCR products obtained with CAPS marker primers (IPM3, IPM4, IPM5, 218R and 73L) were digested with corresponding restriction enzymes (see text, and Table 1) prior to gel electrophoresis. Allele-specific PCR products (111R, 111L and 221R) were subjected to an agarose-gel electrophoresis without digestion. The *arrow(s)* identifying the DNA fragment(s) linked in *cis* to *Rx* are indicated on the right of each panel. DNA size markers are indicated as '*M*'

population, 117L and IPM3 markers were separated from *Rx* by two and three recombination events respectively whereas the GP34 marker, present in BAC167, was separated from *Rx* by 13 recombinations (Fig. 3). The BAC library did not contain additional BACs extending further towards *Rx* from the 191L marker.

Characterization of the BAC clones linked to the IPM4 CAPS marker

The IPM4 marker has been mapped at 0.06 cM from *Rx* on the side away from IPM3 (Bendahmane et al. 1997). Screening of the BAC library with IPM4 identified two clones, BAC73 and BAC111, with inserts of approximately 70 kb each (Fig. 1). The *Taq*I digestion of the IPM4 CAPS marker in these clones indicated that BAC111 was linked in *cis* to the *Rx* locus whereas BAC73 carries a DNA insert from the *rx* chromosome (Fig. 2 b).

To determine the relative genome position of BAC111 and BAC73 we performed PCR tests using end-sequence primers of these BAC clones (Table 1). The results of these tests suggested that BAC73 overlaps with BAC111 and that 73L and 111L represent opposite ends of these BACs. Both 73L and 111L,

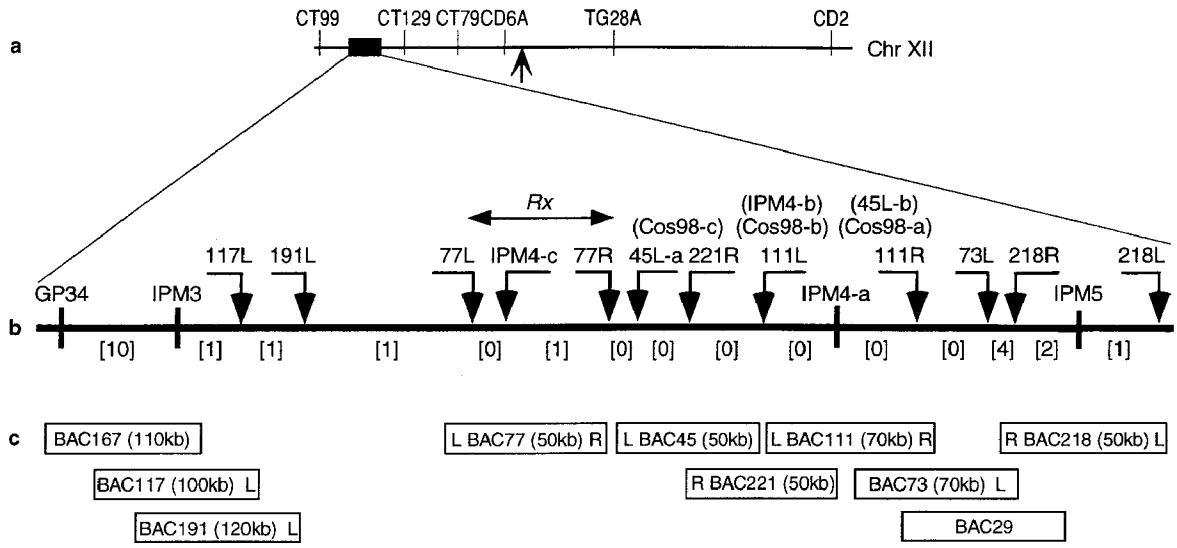


Fig. 3a–c High-resolution map of the *Rx* locus (not drawn to scale). Panel **a** shows a simplified genetic map of potato chromosome XII (denoted by a *horizontal line*) in which the area left of the *arrow* is reversed in the potato and tomato genetic maps (Tanksley et al. 1992). *Vertical lines* indicate positions of previously mapped RFLP markers (Tanksley et al. 1992; Bendahmane et al. 1997). The *filled rectangle* denotes a genetic interval between markers GP34 and 218L, which is magnified in panels **b** and **c**. Panel (**b**) shows the genetic map of the GP34–218L interval (denoted by a *horizontal line*). Positions of the RFLP marker GP34 and the AFLP markers IPM3, IPM4-a and IPM5 are indicated by *vertical lines*. The positions of BAC end-derived markers are indicated by *arrows*. The positions of markers enclosed *in parentheses* (e.g. 45L-b) have not been determined precisely. The *numbers* in square brackets indicate the numbers of S1 Cara individuals containing recombination events in each marker interval. The predicted position of *Rx* is shown by a ' ' symbol. Panel (**c**) shows the positions of BAC clones in the GP34–218L interval. Each *open rectangle* represents one BAC insert DNA. Inside of each rectangle is the name of the BAC clone, the estimated insert size in kb (except for BAC29) and a symbol '*L*' or '*R*' denoting the BAC ends that had been mapped relative to *Rx*

co-segregated with IPM4. In our mapping population of 1720 individuals these markers were both separated from *Rx* by one recombination event (in individual #761) and it was not possible to determine directly which of these markers was physically closer to *Rx*. Hence, to orientate these BACs relative to *Rx*, we screened the BAC library with CAPS markers 111L and 73L. We also screened the BAC library with the IPM5 CAPS marker, which is on the same side of *Rx* as IPM4 but further from *Rx* (Bendahmane et al. 1997). We anticipated that BACs containing IPM5 would allow us to orientate the 111L and 73L markers relative to *Rx* (Fig. 3).

These analyses identified BAC218, carrying an allele of IPM5 identified by *Pst*I digestion as being linked in *cis* to *Rx* (Fig. 1; Fig. 2c). The end-sequences of BAC218 insert DNA were converted into the CAPS markers 218L and 218R, and mapped genetically to the recombination events between GP34 and IPM5. Marker 218L was positioned at eight recombination events (0.48 cM) from *Rx*, between IPM5 and CT129. The 218R marker was positioned between IPM4 and IPM5 at five recombination events (0.30 cM) from *Rx*. We also identified a single BAC pool #29 which contains three markers: 218R, 73L and 111R. CAPS analysis revealed that each of these markers in the BAC pool #29 is represented by the allele linked in *cis* to *Rx* (Fig. 2d). As it is very unlikely ($P = 0.05$) that a single BAC pool contains two different BAC clones carrying markers 218R and 73L or 218R and 111R separately we conclude that BAC pool #29 contains a single BAC clone, BAC29, with a DNA insert linked in *cis* to *Rx*. Therefore, BAC29 provided a link between BAC218 and the IPM4 BAC contig and allowed us to orientate the markers from the IPM4 contig in the following order: *Rx*, 111L, IPM4, 73L (Fig. 3).

By screening the BAC library with 111L allele-specific primers we identified BAC221 which carries an insert DNA of 50 kb and is linked in *cis* to *Rx* (Fig. 1; Fig. 2e). The left end-sequence of BAC221 is located within BAC111 whereas the right end-sequence of BAC221 extends further towards *Rx* (Fig. 3). However the marker 221R co-segregated with IPM4 (Fig 3) in our mapping population and was separated from *Rx* by the recombination event in plant #761.

To extend the IPM4 contig further towards *Rx* we screened the BAC library with 221R allele-specific primers and identified BAC45 which has an insert DNA of 50 kb and is linked in *cis* to *Rx* (Fig. 1; Fig. 2f). The right end-sequence of BAC45 is located inside of BAC221, whereas the left end-sequence of BAC45, 45L, extends further towards *Rx* (Fig. 3). However, BAC45 does not contain *Rx* since the CAPS marker 45L is genetically separated from *Rx* by the recombination event in plant #761. Additional PCR screening of the BAC library with the 45L marker failed to identify any

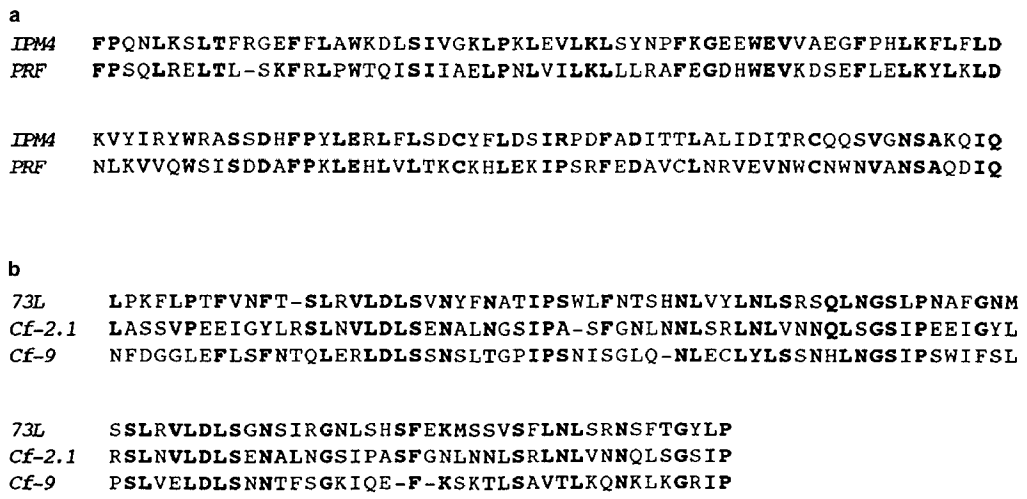


Fig. 4a, b Alignments of putative peptide sequences derived from *Rx*-linked markers. **a** The aligned putative peptide sequence of the IPM4 marker with the homologous region of Prf (Salmeron et al. 1996), and **b** the putative peptide sequences of the 73L marker aligned with homologous regions of the Cf-2 and Cf-9 proteins (Jones et al. 1994; Dixon et al. 1996). Sequences were aligned using BLAST (Altschul et al. 1990). Identical residues are indicated in *bold*, similar residues are in *black*, and different residues are in *grey*

new BAC clones, therefore leaving a gap between the IPM3 and IPM4 BAC contigs (Fig. 3).

Analysis and utilization of duplicated DNA sequences for mapping of *Rx*

The translated versions of several BAC-derived markers from IPM4 contig are similar to cloned disease resistance genes (Fig. 4, and data not shown). The marker IPM4 was similar to the tomato *Prf* gene required for the resistance to *Pseudomonas syringae* pv. *tomato* (Salmeron et al. 1996), and the marker 73L showed a significant degree of homology to the *Cf-2* and *Cf-9* resistance genes of tomato (Dixon et al. 1996; Jones et al. 1994) (Fig. 4). These data indicated that *Rx* is in a cluster of disease resistance-like genes.

Disease resistance loci in plants are often highly complex with small families of resistance genes clustered within tens of kilobases (Ellis et al. 1995; Hulbert and Bennetzen 1991; Jones et al. 1994; Martin et al. 1993; Parniske et al. 1997; Witham et al. 1994). These clusters are due to DNA duplications that may reflect a mechanism of resistance gene evolution (Anderson et al. 1996; Song et al. 1997). Taking into account these findings, we have tested for the presence of the duplicated sequences related to CAPS markers from the vicinity of *Rx* (IPM3–IPM5 interval). In these tests we used pools of DNA from 20 resistant plants (R pool) and 20 susceptible plants (S pool) and the individual BAC clones from the IPM4 contig as templates for PCR amplifications. The primer annealing temperature in PCR reactions was 5 to 10°C lower than in conditions originally developed for each CAPS primer pair (Table 1) so that there would be amplification of the original marker and related homologues. The PCR products obtained with many CAPS primer pairs were the same size as the products produced under high stringency conditions (data not shown). However in three instances digestion of the low stringency PCR products with either *Taq*I, *Alu*I or *Dde*I restriction enzymes revealed several new DNA fragments that were not identified previously. These included fragments that were nonpolymorphic as well as fragments polymorphic between the R and S pools.

The new markers revealed with 45L and cos98 primers all mapped within the BAC contig described above and were not useful for isolation of *Rx* (Fig. 3 and data not shown). However low stringency PCR with the IPM4 primers did reveal a new marker that was more tightly linked to *Rx* than any other of the previously identified markers. *Taq*I digestion of the low stringency IPM4 products from the R pool identified the original IPM4 locus (IPM4-a) in BAC111 and two other restriction fragments that had not previously been detected (Fig. 5a). One of these fragments (IPM4-b) was nonpolymorphic in the R and S pools. This fragment must have originated from BAC221 as the *Taq*I restriction fragment of similar size was also detectable after digestion of IPM4 marker derived from this BAC (Fig. 5a). However, the second new DNA fragment was polymorphic between the R and S pools and was not detected after digestion of IPM4 markers in BAC111 or BAC221 (Fig. 5a). This fragment segregated with *Rx* in all plants of our mapping population including plant #761 and was designated IPM4-c (Fig. 5b).

The potato BAC library was screened by PCR with IPM4 primers using conditions for the detection of IPM4-c. One new BAC clone with a DNA insert of about 50 kb, BAC77, carrying the IPM4-c locus, was identified (Fig. 5a). The end-fragments of the BAC77 DNA insert were cloned, sequenced and converted into the CAPS markers, 77L and 77R. Marker 77L cosegregated with both IPM4-c and *Rx*, whereas 77R was separated from *Rx* by one recombination event in the recombinant individual #761 (based on an analysis of 1720 segregants; see Figs. 3 and 6).

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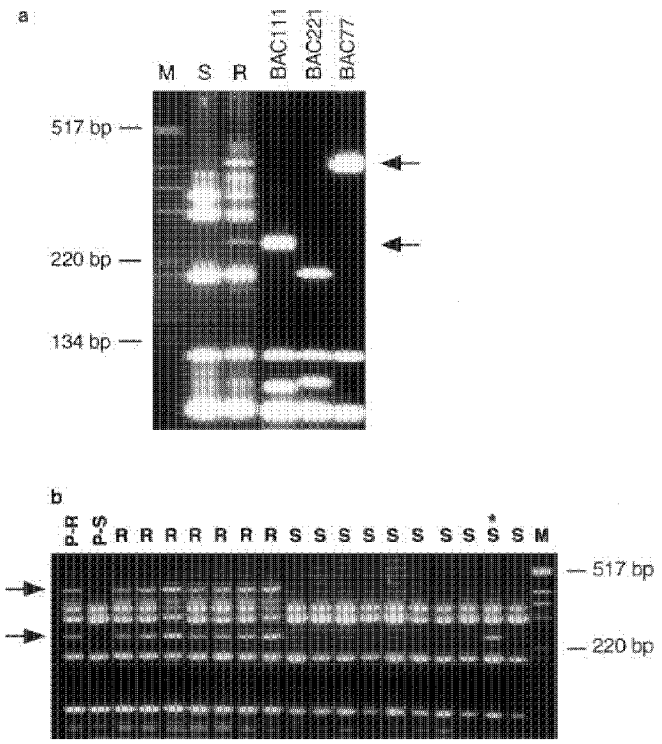


Fig. 5a, b Identification of duplicated sequences in the vicinity of *Rx*. Panel **a** displays the restriction enzyme digestions of the low-stringency PCR products (see text and Table 1) from the R DNA pool (*R*), the S DNA pool (*S*) and BAC DNAs using the IPM4 marker primers. Polymorphic DNA fragments linked in *cis* to *Rx* are indicated by *arrows*. The DNA fragments linked in *cis* to *Rx* but non-polymorphic between R and S pools in the conditions of this experiment are indicated by *double-line arrows*. '*M*', the 1-kb ladder markers (Life Technologies, UK). Panel **b** shows the analysis of individual plants [resistant to PVX (*R*) or susceptible (*S*)] with recombination events in the GP34–IPM5 interval using the IPM4 CAPS marker produced under low-stringency PCR conditions and after digestion with *Taq*I. The R pool and S pool are indicated as '*P-R*' and '*P-S*' respectively, and plant #761 with a recombination event between IPM4-a and IPM4-c/*Rx* is indicated by *a star*. *Arrows* show the positions of IPM4-a- (*lower arrow*) and IPM4-c-specific (*upper arrow*) *Taq*I fragments

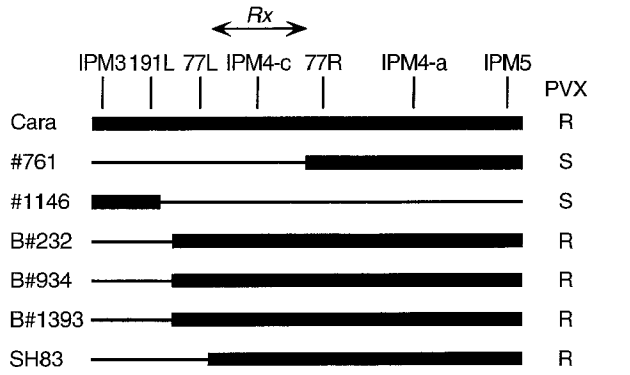


Fig. 6 Introgressed DNA in the vicinity of *Rx*. The diagrams show chromosomal DNA in the IPM3–IPM5 interval from potato cv Cara and clone SH83, and from individuals in our S_1 Cara mapping population (identified in left hand column). These plants were either *Rx* (*R*) or *rx* (*S*) genotypes and the chromosomal region introgressed from *S. tuberosum* spp. *andigena* is shown as a *closed rectangle*. The *S. tuberosum* spp. *tuberosum* DNA is shown as a *thin line*. DNA markers used to map the introgressed DNA are identified over the *vertical lines*, and the region containing *Rx* is delineated by the *horizontal arrow*

Assignment of *Rx* to BAC77 by introgression mapping

To ascertain whether BAC77 spans *Rx*, an additional 1438 individuals from the S1 Cara population were screened with markers 77L, IPM4-c and the flanking markers 191L and 77R (data not shown). There were no plants with recombination events between *Rx* and markers 77L and/or IPM4-c. However, three individuals, B#232, B#934 and B#1393, were found with recombination events in the 191L–77L interval. All of these plants contained *rx*-linked alleles of 191L but were resistant to PVX and also contained the *Rx*-linked allele of 77L (data not shown, Rouppe van der Voort et al. 1998). These data suggest a tight linkage between *Rx* and the marker 77L (Fig. 3) but did not allow *Rx* to be mapped precisely in the 191L–77R interval.

We obtained confirmation that BAC77 spans *Rx* by 'introgression mapping' experiments carried out in a parallel project on the mapping of the *Globodera pallida* resistance gene *Gpa2*. Like *Rx*, this gene resides on the short arm of the potato chromosome XII (Rouppe van der Voort et al. 1997). Both *Gpa2* and *Rx* were introgressed into cultivars Cara and Amaryl from the *S. tuberosum* ssp. *andigena* accession CPC1673 and diploid potato clone SH83-92-488 (referred to hereafter as 'SH83'). SH83 is derived from the cultivar Amaryl and carries both *Rx* and *Gpa2* (Rouppe van der Voort et al. 1998). To map the CPC1673-introgressed DNA in cv Cara, cv Amaryl and in clone SH83, the DNA of these potato genotypes was analysed with CAPS markers in the GP34–IPM5 genetic interval. This analysis provided evidence that all these potato genotypes contain DNA introgressions from the *S. tuberosum* ssp. *andigena* accession CPC1673 within the GP34–IPM5 interval. However, the size of introgressed DNA segments varied between these genotypes (Fig. 6). CPC1673 and cv Cara contain *Rx*-linked alleles of each CAPS marker within the GP34–IPM5 interval. However, in cv Amaryl and clone SH83, the alleles originated from the CPC1673 clone and, linked in *cis* to *Rx*, were confined to the IPM4-c–IPM5 interval (Figs. 6 and 3, and data not shown). The markers in the interval 77L–IPM3 were derived from the PVX-susceptible progenitors of cv Amaryl and clone SH83. Therefore, the left border of the DNA introgressed into these potatoes from CPC1673 is between 77L and IPM4-c (Fig. 3). When combined, the introgression mapping and recombination analyses show that *Rx* is located between markers 77L and 77R (Fig. 6) in the vicinity of IPM4-c.

Discussion

In this report we describe the isolation of a BAC clone which, according to genetic mapping criteria, spans the *Rx* locus in cv Cara. We encountered several issues in the course of isolating this clone that may be relevant to other map-based or marker-assisted cloning projects. One of these issues concerns the frequency of recombination in regions of introgressed DNA. Previous studies have reported a low frequency of recombination in DNA that has been introgressed from alien species (Liharska et al. 1996). Obviously such an effect could hinder map-based cloning of genes in introgressed DNA. However, in the region of DNA that has been introgressed into Cara from *S. tuberosum* ssp. *andigena* accession CPC1673 there is considerable variation in the frequency of recombination. There are certainly some regions, including the region containing *Rx* from 77L to 73R, in which recombination is rare. In our mapping populations of 3150 progeny we detected only two recombination events in that interval of more than 170 kb. However, in other regions, including those spanned by BAC167 and BAC29, the recombination frequency is much higher. In BAC167 the difference was particularly striking because there were ten recombinations within 110 kb of chromosomal DNA, corresponding to approximately 180 kb cM^{-1}. Overall the recombination frequency in potato DNA is higher than 750 kb cM^{-1} (Tanksley et al. 1992).

Initially the presence of repeated DNA sequences around the *Rx* locus complicated the characterization of BAC clones and the construction of BAC contigs. For example, we isolated several BAC clones from our library that were not from the *Rx* chromosome although they had been initially screened with allele-specific PCR or CAPS markers (A.B., K.K. and D.C.B., unpublished). We now know that this anomaly is due to differential PCR in genomic (high-complexity) and cloned (low-complexity) DNA. Repeated DNA loci that are not PCR-amplified from a genomic DNA template may get amplified from cloned DNA because the primer-complementary sequences are more abundant in the cloned DNA than in genomic DNA and because there is no potential for competition for the primers between similar loci.

The complications associated with repeated sequences were compounded by the tetraploid nature of the potato genome and the high degree of polymorphism in the constituent genomes. At some loci there were four different alleles (A.B., K.K. and D.C.B., unpublished). There may be an additional complication with the *Rx* locus which is duplicated on chromosomes V and XII (Ritter et al. 1991). However, despite these complications, we have been able to exploit the presence of repeated sequences for the cloning of the *Rx* locus in an approach which may prove more generally useful.

We reasoned that the *Rx* locus, like other resistance gene loci, may comprise duplicated blocks of resistance genes and that these resistance gene homologues and sequences adjacent to the resistance genes in *Rx*-linked BACs might be duplicated elsewhere in the vicinity of *Rx*. To reveal the other resistance gene homologues we employed low-stringency PCR. This approach is different from the identification of resistance gene homologues using highly degenerate primers based on conserved sequence motifs in resistance genes (Kanazin et al. 1996; Leister et al. 1996, 1998). In the experiments described here we deliberately chose the primers so that these conserved sequence motifs were avoided. It was intended that the low-stringency PCR would be selective for genes at the *Rx* locus rather than homologues located elsewhere in the genome. A future approach to the cloning of resistance genes could exploit both conserved sequence motifs and locus-specific motifs. The highly conserved motifs could be used for rapid identification of resistance gene homologues that are linked to the locus of interest. Low-stringency PCR with non-degenerate primers from other regions of these genes could then be used to identify markers within the resistance locus that are closer to the gene and that could be mapped in a large population.

To demonstrate that BAC77 spans *Rx* it was necessary to identify chromosomal recombinations between *Rx* and each end of this BAC. On the right side, despite the low frequency of recombination in that region, we identified a plant, #761 in our mapping population, with a chromosomal recombination between 77R and *Rx* (Fig. 3). We did not find progeny with chromosomal recombination on the left side between *Rx* and 77L. However in cv Amaryl, in which *Rx* had been introgressed from the same source as in our mapping population, there was a boundary of the introgressed DNA in the *Rx*–77L interval (Fig. 6). We are currently transforming the DNA from this interval (BAC77) into an *rx* cultivar of potato to confirm the presence of *Rx*.

Clearly, marker-assisted cloning of disease resistance genes is now feasible even from plants with complex genomes. However, based on our experience with *Rx*, we can suggest several technical points that should be considered in other similar projects. These include relatively trivial points, such as the construction of BAC libraries with several different restriction enzymes in an attempt to improve representation in the BAC library. In our present large library constructed with *Hin*dIII there were no clones from the 191L–77L region. We can also recommend the use of introgression mapping where several cultivars have been produced carrying the gene of interest introgressed from a single source. Low-stringency PCR may also be useful for complex loci as an approach to the focused identification of new markers. However, even with these combined approaches, there is always the possibility that the gene of interest will be within an interval that is physically large but impossible to dissect by recombination. In

these situations it may be necessary to combine genetical analysis with complementation using transient assays for the trait of interest (Mindrinos et al. 1994) or stable transformation with binary BAC vectors for the assay of long stretches of DNA (Hamilton 1997).

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Development of SCAR markers to the PVY resistance gene Ry_{adg} based on a common feature of plant disease resistance genes

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Abstract: Sequence-characterized amplified regions (SCARs) were developed, based on nucleotide differences within resistance gene-like fragments isolated from a potato plant carrying the Ry_{adg} gene, which confers extreme resistance to potato Y potyvirus (PVY). It originates from *Solanum tuberosum* subsp. *andigena*, and a susceptible potato plant. SCARs were tested using 103 potato breeding lines and cultivars with diverse genetic backgrounds derived from Europe, North America, and Japan. Two markers showed high accuracy for detection of the Ry_{adg} gene. The SCAR marker RYSC3 was generated only in genotypes carrying Ry_{adg}. The SCAR marker RYSC4 was detected in all genotypes carrying Ry_{adg} but also in four PVY-susceptible genotypes. Neither marker was detected in genotypes carrying other *Ry* genes originating from different species than *S. tuberosum* subsp. *andigena*. Therefore, these SCAR markers should be powerful tools in marker-assisted selection for Ry_{adg} in potato breeding programs, and should also be useful for cloning of the Ry_{adg} gene.

Key words: SCAR, potato virus Y, extreme resistance gene, *Solanum*.

Résumé : Des marqueurs SCAR («Sequence-characterized amplified regions») ont été développés en s'appuyant sur des différences nucléotidiques entre fragments d'ADN ressemblant à des gènes de résistance. Ces séquences candidates avaient été isolées d'un plant de pomme de terre sensible et d'un autre possédant le gène de résistance Ry_{adg}, lequel confère une résistance extrême au virus Y de la pomme de terre (PVY) et provient du *Solanum tuberosum* subsp. *Andigena*. Ces séquences candidates ont été testées sur 103 lignées d'améliorateur et cultivars ayant des pedigrees différents et provenant d'Europe, d'Amérique et du Japon. Deux marqueurs se sont révélés très efficaces pour la détection du gène Ry_{adg}. Le marqueur SCAR RYSC3 a été produit uniquement dans les génotypes porteurs de Ry_{adg}. Le marqueur RYSC4 a pour sa part été détecté dans tous les génotypes portant Ry_{adg} mais également chez quatre génotypes sensibles au PVY. Aucun des deux marqueurs n'a été détecté chez des génotypes portant d'autres gènes *Ry* provenant d'espèces autres que le *S. tuberosum* subsp. *andigena*. Ainsi, ces marqueurs SCAR devraient s'avérer de puissants outils en sélection assistée pour le gène Ry_{adg} dans le cadre de programmes d'amélioration variétale ainsi qu'en vue du clonage du gène Ry_{adg}.

Mots clés : SCAR, virus Y de la pomme de terre, gène de résistance extrême, *Solanum*.

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Introduction

Potato virus Y (PVY) is one of the most important viruses in potato and other species of Solanaceae, including tomato, tobacco, and pepper (De Bokx and Huttinga 1981; Matthews 1991). PVY is often transmitted by aphids in a non-persistent manner and causes a heavy yield reduction in most potato-growing areas, especially in Northern Europe, Russia, and North America (Hooker 1981). The most efficient protection against viral disease is achieved by breeding virus-resistant potato cultivars (Swiezynski 1994).

The genetic background for resistance breeding of the cultivated potatoes can be extended by using a number of wild relative species that are available as precious sources of resistance to various biotic stresses (Ross 1986). Thus, germplasm enhancement with diverse wild potato genetic resources should be of value in incorporating valuable resistance traits into the cultivated potatoes (Watanabe et al. 1995). Various resistance genes to PVY have been found in potato and its wild relatives (Valkonen et al. 1996).

Ry genes in *Solanum tuberosum* subsp. *andigena* (Ry_{adg}) and *S. stoloniferum* (Ry_{sto}) are known to provide high level of resistance to PVY (Barker 1997; Hämäläinen et al. 1997). *Ry* genes have been genetically localized on chromosome XI (Brigneti et al. 1997; Hämäläinen et al. 1997). However, the

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selection of the target traits by genetic markers is yet inefficient due to the lack of markers linked to useful traits in the tuber-bearing solanums, including the cultivated potatoes. These problems require conventional potato breeding programs to have large population sizes for selection and causes high cost, high demand for labor, and time to achieve good genetic efficiency (Ross 1986; Watanabe et al. 1995).

Sequence analyses of the cloned plant *R* genes have revealed that *R* genes in different plant species share common features such as leucine-rich repeats (LRRs), leucine zippers and nucleotide binding sites (NBS) (Bent et al. 1994; Jones et al. 1994; Mindrinos et al. 1994; Whitham et al. 1994).

As for the cloning of a PVY resistant gene, Hämäläinen et al. (1998) identified two PCR-derived potato genomic DNA fragments, ADG1 and ADG2, highly linked to Ry_{adg}. These PCR products were produced by primers designed from sequence motifs conserved between the resistance gene *N* of tobacco and *RPS2* of *Arabidopsis thaliana* (Leister et al. 1996, Hämäläinen et al. 1998). Sequence analysis revealed 11 differences between ADG2 (355 bp) fragments derived from PVY-resistant genotype 2x(V–2)7 (EMBL Nucleotide Sequence Database acc. No. Y15297) and susceptible genotype 84.194.30 (EMBL acc. No. Y17789) (Sorri et al. 1999).

One of these nucleotide changes was found in the *Bbv*I recognition site among the ADG2 fragments amplified from different potato genotypes, and an amplified polymorphic sequence (CAPS) marker to distinguish genotypes carrying Ry_{adg} from those lacking this gene was developed (Sorri et al. 1999). ADG2 contains the predicted kinase-2 and kinase-3a motifs similar to the homologous regions of *N* and *RPP5*, and the *Bbv*I recognition site is located in kinase-3a motif (Sorri et al. 1999).

Other types of molecular markers can be generated by further analyzing the ADG2 fragment, such as single strand conformation polymorphism (SSCP, Orita et al. 1989) and sequence characterized amplified region (SCAR, Paran and Michelmore 1993). SCAR can be considered as a more useful marker because it is easier to score.

In this study, PCR primers for SCAR were designed based on differences in the nucleotide sequences among ADG2 homologues derived from PVY-resistant and susceptible potato genotypes. Ry_{adg} genotypes and other *Ry* genotypes, as well as PVY-susceptible genotypes were tested using the SCAR markers. The accuracy, efficiency, and feasibility of the use of the SCAR markers developed in this study are discussed, and compared with other types of molecular markers such as RFLP (restriction fragment length polymorphism), RAPD (random amplified polymorphic DNA) and CAPS.

Materials and methods

Plant materials

Plant materials used in this study included common tetraploid potato cultivars, and tetraploid and diploid potato breeding lines with different genetic backgrounds. The haploid ($2n = 2x = 24$) potato clone 2x(V–2)7 containing Ry_{adg}, and the diploid ($2n = 2x = 24$) potato breeding line 84.194.30 were used as controls for extremely resistant and susceptible reaction to PVY infection, respectively (Valkonen et al. 1994*b*). AA–3 ($2n = 4x = 48$) is a sesquiploid–tetraploid hybrid from a cross between tetrasomic tetraploid *S. tuberosum* 7XY.1 ($2n = 4x = 48$) and disomic tetraploid *S. acaule* 954.3CA ($2n = 4x = 48$, Watanabe et al. 1994*b*). TA (*S. tuberosum* × *S. acaule*) genotypes, containing Ry_{adg} originating from a clone of *S. tuberosum* subsp. *andigena*, were backcrossed progeny derived from AA–3 and a diploid pollinator DG81–68, furnishing *2n* pollen (Watanabe et al. 1992, 1997). In addition to potato genotypes carrying Ry_{adg}, we used other potato genotypes that have different genes for extreme resistance to PVY derived from different tuber-bearing species such as *S. chacoense*, *S. phureja,* and *S. stoloniferum*, and the non-tuber-bearing *S. brevidens*. Among the total of 103 genotypes tested in this study, a total of 24 genotypes were extremely resistant to PVY.

Table 1. Nucleotide differences within the ADG2 homologues derived from PVY resistant potato genotype 2x(V–2)7 and susceptible genotype 84.194.30.

	Nucleotide	
Position No.	2x(V–2)7	84.194.30
38	C	T
141	G	T
177	A	G
181	C	T
190	C	G
220	A	G
222	A	T
260	C	G
297	T	A
312	C	T
321	T	C

Preparation of template DNA

Total genomic DNA was extracted from pathogen-free plants grown in vitro using CTAB (cetyltrimethylammonium bromide) methods (Doyle and Doyle 1987). Extracted DNA was treated with RNase at 37°C overnight, and purified by ethanol precipitation (Michaels et al. 1994). DNA samples were diluted to 25 ng/μL with TE buffer and stored at 4°C.

Primer design for SCAR markers

It was reported that there were 11 nucleotide differences between the ADG2 from 2x(V–2)7 and 84.194.30 (Table 1, Sorri et al. 1999). Based on these nucleotide changes, three sets of oligonucleotide primers for SCAR were designed and expected to recognize the ADG2 of 2x(V–2)7 specifically (Table 2). Amplification was simulated on each ADG2 sequence generated from 2x(V–2)7 and 84.194.30, with 16 pairs of primer combinations, including original primers (3.3.3s and 3.3.3as, Hämäläinen et al. 1998), by a PCR simulation program AMPLIFY 1.2 (Dept. Genetics, Univ. Wis., U.S.A.).

PCR conditions

PCR was carried out in a reaction mixture (50 μL) containing 1× PCR buffer (Perkin–Elmer, Foster City, U.S.A.), 0.1 mM each of dNTP (Perkin–Elmer), 1.5 mM $MgCl_2$, 0.25 μM each primer, 1 U Taq polymerase (AmpliTaq Gold, Perkin–Elmer) and 50 ng of template DNA. Amplification was performed in PTC–100 Programmable Thermal Controller (MJ Research, Inc., Watertown, U.S.A.) programmed at 93°C for 9 min, followed by 35 cycles of: denaturation at 94°C for 45 s, primer annealing at 60°C for amplification of RYSC3, or 62°C for amplification of RYSC4 for 45 s, and primer extension at 72°C for 60 s, followed by a final extension at 72°C for 5 min.

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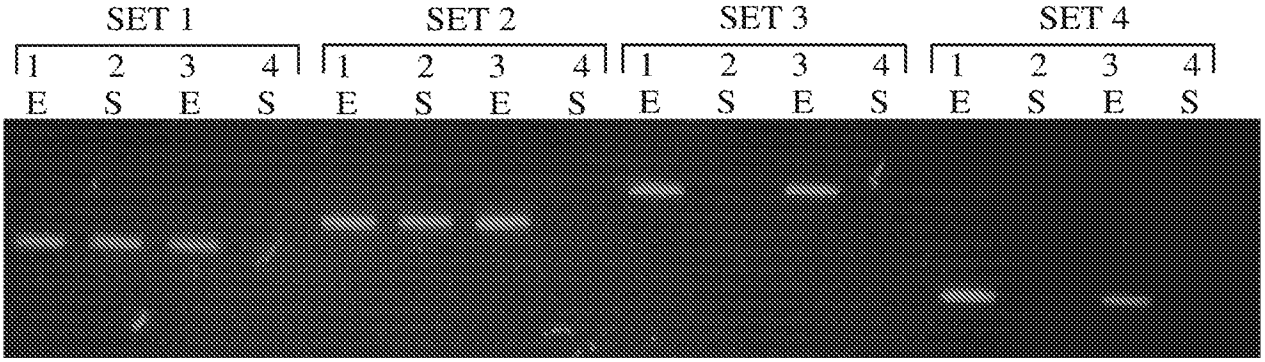
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Table 2. Primers used for PCR amplification of the ADG2 and SCARs.

Primer	Sequence	Position[a]	Length (bp)	Set[b]
3.3.3s	5′-ATA CAC TCA TCT AAA TTT GAT GG-3′	1–23	23	1F, 2F, 3F
3.3.3as	5′-ACT TAA CTG CAT CAT GTT CAA G-3′	334–355	22	–
ADG21F	5′-AGT TCT AGT TGT GCT TGA TAA C-3′	177–197	22	4F
ADG21R	5′-GTT ATC AAG CAC AAC TAG AAC T-3′	(177–197)	22	1R
ADG22R	5′-CAA GAT CCC CTG CTA GGT ATT T-3′	(220–241)	22	2R
ADG23R	5′-AGG ATA TAC GGC ATC ATT TTT CCG A-3′	(297–321)	25	3R, 4R

[a]Position on a complementary strand is shown in parentheses.
[b]F, forward; R, reverse.

Fig. 1. DNA amplification of four potato genotypes using four combinations of SCAR primers derived from ADG2. Resistance phenotypes are indicated above the lanes; E and S indicate extreme resistance and susceptible to PVY, respectively. Lane 1, 2x(V–2)7; lane 2, 84.194.30; lane 3, 7XY.1; lane 4, DG81–68. Lanes 1 and 3 contain PVY resistance gene Ry_{adg}.



Analysis of PCR products

Existence of amplified products was checked electrophoretically on a 2% agarose gel using 1× TAE buffer. After electrophoresis, gels were stained with ethidium bromide, destained with distilled water, visualized with IMAGEMASTER® VDS (Pharmacia Biotech, Uppsala, Sweden) and photographed using Thermal Imaging System (Fujifilm, Tokyo) for data analysis. Reproducibility of PCR experiments was confirmed at least twice.

Results

Identification of primer combination for SCAR markers

Four combinations of primers expected to amplify a PCR product bigger than 140 bp, specific to 2x(V–2)7, were selected and tested using template DNA extracted from 2x(V–2)7, 84.194.30, 7XY.1, and DG81–68. It was shown that primer set 3 (3.3.3s and ADG23R) and set 4 (ADG21F and ADG23R) amplified a single PCR product of 321 bp and 145 bp, respectively, only in the PVY-resistant genotypes 2x(V–2)7 and 7XY.1 (Fig. 1). The PCR products generated by primer set 3 and set 4 were designated as RYSC3 and RYSC4, respectively. In all 5 genotypes, no PCR amplification should be detected by the use of a SCAR primer set.

Applicability test of SCAR markers

Applicability of RYSC3 and RYSC4 as SCAR markers was tested using 103 tetraploid and diploid genotypes with different genetic backgrounds. In these potato genotypes, 14 genotypes have been reported to contain Ry_{adg}, and 10 genotypes have been reported to express extreme resistance genes to PVY controlled by other resistance genes derived from *S. brevidens*, *S. chacoense*, *S. phureja* or *S. stoloniferum* (Table 3).

RYSC3 was present in all 14 genotypes containing Ry_{adg}. It should be noted that reduction of template DNA concentration was required in the case of AA–3, which might be due to its sesquiploid background (Watanabe et al. 1994*b*). No amplification product was detected in susceptible genotypes. Therefore, the amplification of RYSC3 corresponded completely to presence of the Ry_{adg} gene.

RYSC4 was amplified in all genotypes containing Ry_{adg} but it was also detected in 'Papa Amarilla', 'Pentland Ace', 'Pentland Dell', and 'Eniwa', that do not express extreme resistance to PVY. Therefore, the correlation between presence of Ry_{adg} / S and RYSC4 marker was 96.1%.

It should be worth noting that the SCAR markers were generated exclusively with the PVY-resistant lines containing Ry_{adg} derived from *S. tuberosum* subsp. *andigena*.

Discussion

Along with recent exploding advancement of plant molecular genetics, various kinds of PCR-based molecular markers have been developed for plant breeding, such as RAPD (Williams et al. 1990), SCAR (Paran and Michelmore 1993), CAPS (Akopyanz et al. 1992), and AFLP (amplified fragment length polymorphism, Vos et al. 1995). Molecular markers are considered to be an important tool leading to enhanced progress in potato breeding, as well as in breeding of other crop species (Watanabe 1994).

Among the molecular markers developed thus far, SCAR markers seem to be the most valuable (Paran and Michelmore 1993). For example, PCR-based markers do not

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Table 3. Examination of potato cultivars and breeding lines using SCAR markers.

Potato clone	PVY resistance phenotype[a]	Resistance donor species[b]	Ploidy	Presence of the marker band[c]		References to pedigree and PVY resistance
				RYSC3	RYSC4	
2x(V–2)7	E	*adg*	2*x* (haploid)	+	+	Valkonen et al. 1994*b*, Watanabe et al. 1994*a*
$\{2x(V–2)7 \times 84.194.30\}_{62}$	E	adg	2*x*	+	+	Sorri et al. 1999
84.194.30	S		2*x*	–	–	Valkonen et al. 1994*b*, Watanabe et al. 1994*a*
IvP 35	E[d]	phu	2*x*	–	–	Valkonen et al. 1995
DG.81–68	S		2*x*	–	–	Swiezynski et al. 1989
954.3CA	S		4*x*	–	–	Watanabe et al. 1994*b*
AA–3	E	adg	4*x*	+	+	Watanabe et al. 1994*b*, Iwanaga et al. 1991
7XY.1	E	adg	4*x*	+	+	Watanabe et al. 1994*b*, Iwanaga et al. 1991
I12.1	S		4*x*	–	–	E. Fernandez-Northcote, personal communication
BW5.116	S		4*x*	–	–	E. Fernandez-Northcote, personal communication
F1.1	E[d]	chc	2*x*	–	–	Hosaka and Hanneman 1994
84.35.7	S		2*x*	–	–	Watanabe et al. 1994*a*
84.36.29	S		2*x*	–	–	Watanabe et al. 1994*a*
85.37.38	S		2*x*	–	–	Watanabe et al. 1994*a*
86.54.18	E[d]	sto	4*x*	–	–	CIP[e] 1998
86.61.26	E[d]	sto	2*x*	–	–	Valkonen et al. 1994*b*, Watanabe et al. 1994*a*
90.30.47	S		2*x*	–	–	E. Fernandez-Northcote, personal communication
90.31.42	S		2*x*	–	–	E. Fernandez-Northcote, personal communication
acl 7–8	S		4*x*	–	–	S. Slack, personal communication
87HW13.7	S		2*x*	–	–	Valkonen et al. 1995
CPC2451	E[d]	brd	2*x*	–	–	Valkonen et al. 1995
TET38.12	E[d]		2*x*	–	–	Valkonen et al. 1995
TET38.13	H		2*x*	–	–	Valkonen et al. 1995
TET38.2	H		2*x*	–	–	Valkonen et al. 1995
TET38.9	E[d]		2*x*	–	–	Valkonen et al. 1995
E74–7	E	adg	4*x*	+	+	Hämäläinen et al. 1997
HHI–9.3CD	S		4*x*	–	–	E. Fernandez-Northcote, personal communication
M200.38.4CD	S		4*x*	–	–	E. Fernandez-Northcote, personal communication
N140–201	E	adg	4*x*	+	+	Hämäläinen et al. 1997
Q237–8	E	adg	4*x*	+	+	Hämäläinen et al. 1997
S48–6	H		4*x*	–	–	R.L. Plaisted, unpublished
TA1.26.1.1	S		4*x*	–	–	Watanabe et al. 1992, 1997
TA1.27.1.1	S		4*x*	–	–	Watanabe et al. 1992, 1997
TA3.1.1	E	adg	4*x*	+	+	Watanabe et al. 1992, 1997
TA3.3.3	E	adg	4*x*	+	+	Watanabe et al. 1992, 1997
TA3.5.3.6	E	adg	4*x*	+	+	Watanabe et al. 1992, 1997
TA3.8.3.3	E	adg	4*x*	+	+	Watanabe et al. 1992, 1997
All Blue	S		4*x*	–	–	CIP 1998
Allegany	H	adg	4*x*	–	–	Valkonen et al. 1994*a*
Alpha	S		4*x*	–	–	CIP 1998
Andover	S		4*x*	–	–	Plaisted et al. 1998
Atlantic	S		4*x*	–	–	Russo and Slack 1998, Valkonen et al. 1994*a*
Atzimba	S or H		4*x*	–	–	CIP 1998

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Table 3 (*continued*).

Potato clone	PVY resistance phenotype[a]	Resistance donor species[b]	Ploidy	Presence of the marker band[c] RYSC3	RYSC4	References to pedigree and PVY resistance
Bintje	S		4*x*	–	–	Valkonen and Palohuhta 1996, Stegmann and Schnick 1982
Chieftain	S		4*x*	–	–	CIP 1998
Desiree	H	tbr	4*x*	–	–	Jones 1990, Stegmann and Schnick 1982
Garnet Chilli	E[d]	tbr	4*x*	–	–	CIP 1998
Gineke	S		4*x*	–	–	CIP 1998
Greta	S		4*x*	–	–	CIP 1998
Kanona	S		4*x*	–	–	CIP 1998
King Edward	S		4*x*	–	–	Jones 1990, Stegmann and Schnick 1982
M. Hindenburg	S		4*x*	–	–	CIP 1998
Maris Piper	S		4*x*	–	–	Jones 1990, Stegmann and Schnick 1982
Matilda	H	tbr	4*x*	–	–	Valkonen and Palohuhta 1996
Mentor	S		4*x*	–	–	CIP 1998
Norchip	S		4*x*	–	–	CIP 1998
NY99	S		4*x*	–	–	Hämäläinen et al. 1997
NY101	S		4*x*	–	–	CIP 1998
NY103	E	adg	4*x*	+	+	CIP 1998
NY109	S		4*x*	–	–	CIP 1998
NY112	S		4*x*	–	–	CIP 1998
NY115	S		4*x*	–	–	CIP 1998
NY121	E	adg	4*x*	+	+	CIP 1998
NY123	E	adg	4*x*	+	+	CIP 1998
Papa Amarilla	S		4*x*	–	+	CIP 1998
Pentland Ace	S		4*x*	–	+	CIP 1998
Pentland Crown	H		4*x*	–	–	Valkonen et al. 1994*a*, Jones 1990
Pentland Dell	H		4*x*	–	+	Valkonen et al. 1994*a*, Valkonen and Rokka 1998
Pentland Ivory	H	tbr	4*x*	–	–	Jones 1990, Stegmann and Schnick 1982
Pike	S		4*x*	–	–	CIP 1998
Pito	H	tbr	4*x*	–	–	Valkonen 1997
Purple Peravian	S		4*x*	–	–	CIP 1998
Russet Burbank	S		4*x*	–	–	CIP 1998
Russet Rural	S		4*x*	–	–	CIP 1998
Salem	S		4*x*	–	–	CIP 1998
Serrana	H		4*x*	–	–	CIP 1998
Snowden	S		4*x*	–	–	CIP 1998
Stirling	H		4*x*	–	–	CIP 1998
Superior	S		4*x*	–	–	CIP 1998
Yagana	S		4*x*	–	–	CIP 1998
Yukon Gold	H		4*x*	–	–	Valkonen 1997
Japanese cultivars						
Benimaru	S		4*x*	–	–	HKAES[f] 1998
Chidiwa	S		4*x*	–	–	HKAES 1998
Danshaku-Imo	S		4*x*	–	–	HKAES 1998
Dejima	S		4*x*	–	–	HKAES 1998
Eniwa	S		4*x*	–	+	HKAES 1998
Ezoakari	S		4*x*	–	–	HKAES 1998
Hanashibetu	S		4*x*	–	–	HKAES 1998
Hatsufubuki	S		4*x*	–	–	HKAES 1998
Hokkaishiro	S		4*x*	–	–	HKAES 1998

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Table 3 (*concluded*).

Potato clone	PVY resistance phenotype[a]	Resistance donor species[b]	Ploidy	Presence of the marker band[c] RYSC3	RYSC4	References to pedigree and PVY resistance
Kitaakari	S		4*x*	–	–	HKAES 1998
Konafubuki	E[d]	chc	4*x*	–	–	HKAES 1998
May Queen	S		4*x*	–	–	HKAES 1998
Musamaru	S		4*x*	–	–	HKAES 1998
Myoujou	S		4*x*	–	–	HKAES 1998
Norin no. 1	S		4*x*	–	–	HKAES 1998
Ohjiro	S		4*x*	–	–	HKAES 1998
Rishiri	S		4*x*	–	–	HKAES 1998
Sakurafubuki	E[d]	chc	4*x*	–	–	HKAES 1998
Touya	S		4*x*	–	–	HKAES 1998
Toyoshiro	S		4*x*	–	–	HKAES 1998
Waseshiro	S		4*x*	–	–	HKAES 1998
Yukijiro	S		4*x*	–	–	HKAES 1998
Correlation[g]				100.0%	96.1%	

[a]E, extremely resistant; H, hypersensitive; S, susceptible.
[b]adg, *Solanum tuberosum* subsp. *andigena*; brd, *S. brevidens*; chc, *S. chacoense*; sto, *S. stoloniferum*; phu, *S. phureja*; tbr, *S. tuberosum*.
[c]+, marker band detected; –, not detected.
[d]Extreme resistance to PVY not derived from *S. tuberosum* subsp. *andigena*.
[e]International Potato Center, Lima, Peru.
[f]Hokkaido Konsen Agricultural Experimental Station, Japan.
[g]Correlation between presence of Ry_{adg} gene and SCAR marker band.

require large amounts of purified DNA, in contrast to hybridization methods like RFLP. With SCAR markers, scoring the results is more straightforward than other PCR-based markers, such as RAPD and AFLP. In comparison to CAPS markers, the SCAR marker can be revealed by only a single PCR procedure, without subsequent digestion with a restriction enzyme. Another advantage of PCR-based markers is that genomic information can be obtained directly by analyzing PCR products. Consequently, SCAR markers do not only offer the most practical methods for screening numerous samples in a time- and labor-saving manner, but may also offer starting points for cloning the target genes.

SCAR markers developed in this study showed extremely high correlation with the genotypes carrying Ry_{adg}, irrespective of the diverse genetic backgrounds (Table 3). RYSC3, in particular, exhibited a complete match to presence of Ry_{adg} among the total of 103 tested. Another advantage of these SCAR markers was their specificity to genotypes carrying Ry_{adg}, while genotypes containing *Ry* genes not derived from *S. tuberosum* subsp. *andigena* were not detected. These SCAR markers should be powerful tools for detection of genotypes carrying the Ry_{adg} gene in potato-breeding programs because of their high accuracy, feasibility of use, and cost efficiency. Further simplification of the use of the SCAR markers is in progress with the aim of turning these markers into kits suitable for breeding programs (K.N. Watanabe, K. Kasai, and Y. Morikawa, in preparation).

The pronounced accuracy of these SCAR markers in detection of Ry_{adg} further supports the hypothesis that these SCARs and ADG2 might be components of the Ry_{adg} gene (Hämäläinen et al. 1998; Sorri et al. 1999). The hypothesis that ADG2 might be a part of the Ry_{adg} gene was previously presented based on the nucleotide sequence similarity to the corresponding region of the *N* gene in tobacco (Hämäläinen et al. 1998), the presence of kinase-2 and kinase-3a motifs in ADG2, and the finding that the corresponding gene is expressed (Sorri et al. 1999). The SCAR markers RYSC3 and RYSC4 cover the kinase motifs within ADG2.

S. tuberosum subsp. *andigena* also has a virus resistance gene, namely the gene Ra_{adg} for resistance to potato A potyvirus (PVA), located only 6.8 cM from Ry_{adg} towards the distal end of chromosome XI (Hämäläinen et al. 1998). Although resistance to PVA was not examined in this study, our data infer that in breeding programs utilizing Ry_{adg} for resistance to PVY, resistance to PVA controlled by the closely linked locus is likely to be co-segregating. Thus, the SCAR markers of this study may be used for selection for two important resistance traits derived from the same source.

Sequencing of the ADG2-flanking regions appears to be an intriguing way to clone the Ry_{adg} gene. Progress is being made on the isolation of flanking regions of ADG2 by inverse–PCR, and screening potato cDNA and genomic libraries using ADG2 fragment as a probe (J.P.T. Valkonen and S. Vidal, in preparation). Comparison of the sequences of the ADG2 homologues and flanking regions from various genotypes is expected to offer valuable information not only on the resistance gene itself but also on phylogenetic relationships in Solanaceae (K.N. Watanabe, A. Shiranita, K. Kasai and T. Ohdan, in preparation).

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Regulation of Starch Contents in Potato (*Solanum tuberosum* L.) by Manipulation of Sucrose Synthase Gene

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Summary

Transgenic potato microtubers were created in which the expression of sucrose synthase (Susy) was altered by introducing a chimeric gene containing the coding region of Susy that is linked to the patatin promoter in sense (S) or antisense (AS) orientation. The analysis of transgenic microtubers showed that Susy activity and starch content increased in S plants 8, 22, and 30 transformed with the chimeric sense gene; they decreased in AS plants 3 and 10 transformed with the antisense gene. RT-PCR analysis revealed that the level of mRNA for Susy increased in S plants, and decreased in AS plants. There was a little change in the expression of genes for ADP-glucose pyrophosphorylase (AGPase), one of key enzymes in starch biosynthesis. The level of mRNA for AGPase and the activity of the enzyme increased in S 8 plant, but decreased in AS 3 plant.

Key Words: microtuber, patatin promoter, starch content, sucrose synthase, transgenic potato.

Introduction

Starch is the major reserve carbohydrate in most higher plants. It is stored in the form of water-insoluble, osmotically inactive granules in amyloplasts and chloroplasts, which are the major subcellular organelles where starch biosynthesis takes place. Starch synthesis within the amyloplasts is completely dependent on the import of carbon from the cytosol. The bulk of man's carbohydrate intake arises from the consumption of starch storage organs such as seeds of cereals and tubers of potato. Potato, an important tuberous crop stores starch in the amyloplasts.

As sucrose is the major sugar that is translocated from leaves to developing potato tubers, the mechanisms responsible for the immediate metabolism of sucrose in tubers are of particular interest. Sucrose, transported to plant sink organs, is generally converted into monosaccharides or derivatives. Sucrose can be degraded by two different enzymes, sucrose synthase (EC 2.4.1.13; Susy) or invertase (Morrell and ap Rees, 1986). Susy catalyzes a rapid reversible reaction between sucrose and UDP into UDP-glucose and fructose in developing potato tubers and other plant tissues (Geigenberger and Stitt, 1993); it has been proposed that Susy activity can be used as a parameter for sink strength (Sung et al., 1989).

Susy, ubiquitous in higher plants (Avigad, 1982), plays a variety of important roles. As the predominant sucrose cleavage enzyme in the cereal endosperm and potato tuber; it catalyzes substrates for starch synthesis (Chourey and Nelson, 1976; Claussen et al., 1985; Dale and Housley, 1986; Sung et al., 1989). Measurements of maximum catalytic activities showed that only Susy activity could sustain a rate of sucrose breakdown, comparable with that observed *in vivo* (Morrell and ap Rees, 1986). Thus, it is likely that the first step in the conversion of sucrose to starch is largely catalyzed by Susy.

A cDNA clone for Susy in potato tubers has been isolated and characterized by Salanoubat and Belliard (1987). Zrenner et al. (1995) used transgenic potato plants to show that Susy is the major sucrose-metabolizing enzyme in growing potato tubers. To further investigate the influence of Susy on the starch biosynthesis of potato tubers, we tried to change the expression level of Susy gene by using sense/antisense RNA approach. In addition, we tried to produce transgenic potatoes with increased starch content by using Susy gene manipulation for a practical purpose.

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Materials and Methods

Plants, bacterial strains and growth conditions

Potato plants (*Solanum tuberosum* L. cv. Desiree) in tissue culture were maintained under a 16hr light (30 $\mu mol \cdot m^{-2} \cdot s^{-1}$)-8 hr dark regime (23 ℃) on MS medium, containing 30 $g \cdot liter^{-1}$ sucrose, Staba vitamin,

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100 mg·liter^{-1} inositol, pH 5.8, solidified with 2 g·liter^{-1} Phytagel (Sigma Co. LTD). Microtubers were produced according to the method described by Joung (1989). Nodal cuttings, taken from the shoot, were placed on the medium as described above except that sucrose was increased to 90 g·liter^{-1}. Growth conditions for *in vitro* tuberization were at 18 ℃ under an 8 hr light (7 μmol·m^{-2}·s^{-1})-16 hr dark regime.

Escherichia coli strain DH5α was cultured using standard techniques (Sambrook et al., 1989). *Agrobacterium tumefaciens* strain LBA4404, containing pBI121, was cultured in YEP medium (1.0% bacto-peptone, 1.0% yeast extract, and 0.5% NaCl).

DNA manipulation

DNA manipulation was done essentially as described by Sambrook et al. (1989). The gene for Susy was amplified from potato cDNAs, using polymerase chain reaction (PCR). The sequence of primers was designed, based on the Susy cDNA (2417bp) published by Salanoubat and Belliard (1989). The sequence of Susy1 primer was 5'-dATGGCTGAACGTGTTTTGACTCGT-GTTCAT-3' and that of Susy2 primer was 5'-dACT-CAGCAGCCAATGGAACAGCTTCAGCCA-3'. The Susy gene was amplified by a fill-in PCR reaction (denaturation, 94 ℃-1 min; annealing, 60 ℃-1 min; polymerization, 72 ℃-2 min; 35 cycles) with PWO polymerase (Boehringer Manheim Co., Germany) and cloned into the *Eco*RV site of pT7Blue (Novagene Co.), creating pT7BlueSS2. To increase/reduce the Susy activity in transgenic potato plants, the 2.4kb cDNA encoding Susy of potato was cloned in sense/antisense orientation between the patatin promoter and the terminator of nopaline synthase in the binary vector pBI-PAT, which was derived from pBI121 (Fig. 1). Here the Susy gene is under the control of a patatin promoter for tuber-specific expression. The sequence and orientation of the sense construct (pBI-PAT-S) and the antisense construct (pBI-PAT-AS) were confirmed by Silver sequencing (Promega Co.) and digestion by *Xba*I and *Eco*RI.

Plant transformation

Vectors pBI-PAT-S and pBI-PAT-AS were used to transform potato plants by using *Agrobacterium*-mediated gene transfer as described by Rocha-Sosa et al. (1989).

RT (Reverse transcriptase)-PCR

Total RNA was extracted by the Ultraspec^{TM}-II RNA isolation system (Biotex Inc., Houston Tx, USA), according to the supplier's manual.

First-strand cDNA was synthesized from total RNA with Susy2 primer and AMV reverse transcriptase. Synthesized cDNA was purified, then tailed with dATP and terminal transferase. The dA-tailed cDNA was amplified by PCR (denaturation, 94 ℃-15 sec; annealing, 55 ℃-30 sec; polymerization, 72 ℃-40 sec; 10 cycles). The reaction mixture (50 μl) contained 0.2mM dNTPs, 2.5 units of *Taq* DNA polymerase, 50 pmoles of oligo dT-anchor primer, and Susy2 primer, and 1 μg of dA-tailed cDNA.

The probe was prepared by PCR with the DIG-labeling kit (Boehringer Mannheim Co., Germany). The primers were Susy1 and Susy2; the template was pT7BlueSS2. The PCR conditions were the same as above. The following cDNA clones were also used for labeling reactions: AGPase B, EcoRI insert of plasmid B22-1 (Müller-Röber et al., 1990) and AGPase S, *Eco*RI insert of plasmid S25-1 (Müller-Röber et al., 1990).

After electrophoresis, the amplified cDNA was transferred to a positively charged nylon membrane by using Turboblotter rapid downward transfer systems (Schleicher & Schuell, Co, Keene, NH, USA.). Transferred DNA was crosslinked to the membrane by UV-light and prehybridized with standard hybridization solutions (5×SSC, 1% blocking reagent, 0.1% N-lauroyl sarcosine Na-salt, 0.02% SDS). DIG-labeled DNA probe was denatured and allowed to hybridize. The membrane was washed and then used directly for detection of hybridized DNA by using a DIG DNA

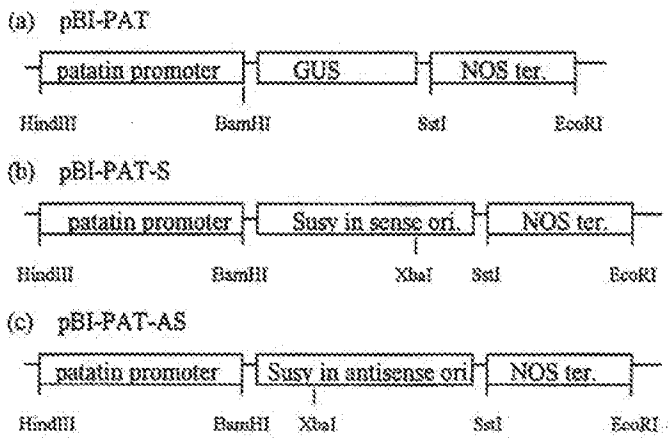


Fig. 1. Construction of chimeric genes for expression of sucrose synthase sense and antisense RNA.

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detection kit (Boehringer Mannheim Co., Germany).

Susy and ADP-glucose pyrophosphorylase (AGPase) assay

Two grams of fresh microtubers were homogenized in a 2ml extraction buffer A (50 mM Tris-HCl, 10 mM glutathione, 1 mM EDTA, 20 mM $NaHSO_4$, pH7.5) and centrifuged at 26,000 × g for 10 min at 4 ℃. The supernatant was dialyzed overnight against 40 volumes of the extraction buffer with one change, and used to assay for enzyme activity. The reaction mixture contained 40 mM Tris-phosphate (pH 6.6), 250 μM sucrose, 10 μM UDP, 5 μM mercaptoethanol, 10 μM NaF, and a diluted extract (0.1 mg protein) in a final volume of 1ml. The enzyme reaction was conducted at 37 ℃ for 2 min and stopped by heating at 95 ℃ for 3 min. The fructose liberated was determined by an analysis kit (Boehringer Mannheim Co., Germany). One unit of enzyme activity is defined as that which liberates 1 μmole of fructose per hour. The Susy activity was corrected by subtraction of the control value from a reaction mixture prepared without UDP.

Several pieces of microtuber slice (total 1 g) were homogenized in a 1ml extraction buffer B (50 mM Hepes-KOH; pH7.4, 5 mM $MgCl_2$, 1 mM EGTA, 1 mM EDTA, 10% glycerol, 0.01% Triton X-100). After centrifugation, the supernatant was assayed for AGPase. The production of glucose-1-phosphate from ADP-glucose was determined in an NAD-linked glucose-1-phosphate dehydrogenase system (Plaxton and Preiss, 1987). The reaction mixture contained 80 mM HEPES (pH 7.4), 10 mM $MgCl_2$, 1 mM ADP-glucose, 0.6 mM NAD, 10 μM glucose-1,6-bisphosphate, 3 mM DTT, 0.02% BSA, 1U phosphoglucomutase, 2.5U NAD-linked glucose-6-phosphate dehydrogenase, and tuber extract (0.1 mg protein) in a final volume of 1ml. The reaction was initiated by the addition of sodium pyrophosphate. The NAD reduction was measured spectrophotometrically at 340nm.

Determination of soluble sugars and starch

Contents of starch, sucrose, glucose, and fructose were determined by the sucrose/D-glucose/D-fructose determination kit and starch determination kit of Boehringer Mannheim Co. (Germany).

The fresh weight of microtubers was determined immediately after harvest. For determination of dry weight, randomly selected microtubers were sliced and dehydrated at 65 ℃ until the weight decreased no further.

Results and Discussion

Screening of transgenic plants

To regulate Susy activity in transgenic potato plants via sense and antisense RNA approaches, two gene constructs; pBI-PAT-S for sense expression of Susy and pBI-PAT-AS for antisense inhibition of Susy (Fig. 1) were used to transform potato plants.

After transformation 214 kanamycin-resistant shoots with Susy sense gene and 177 kanamycin-resistant shoots with Susy antisense gene grown in tissue culture were selected. Crude DNA was extracted from all regenerated plants and used as a template for PCR detection of NPTII (neomycin phosphotransferase) gene (Wang et al., 1993). By PCR screening, we obtained 20 transgenic plants with pBI-PAT-S (subsequently called S plants) and 7 transgenic plants with pBI-PAT-AS (AS plants).

In vitro propagated transgenic plants were morphologically indiscernible from the non-transformed control plants; there was no phenotypical difference in the number of microtubers formed per stolon (data not shown). These observations indicate that an alteration of the Susy activity does not change the growth habit.

Analysis of the expression of Susy in transgenic potato plants

The non-transformed control plants, control plants transformed with pBI-PAT and transgenic S, and AS plants were allowed to form microtubers. These microtubers were harvested and analyzed for the Susy gene expression and enzyme activity. The RT-PCR analysis, using the Susy cDNA as a probe, revealed high levels of Susy mRNA in the S8, S22, and S30 plants, and their Susy activities were 11.9, 12.5 and 12.3 units·mg $protein^{-1}$, respectively, or about 1.5-fold more than that of the control (Table 1). These values correspond to 138-145% of the control activity in another experiment (Fig. 2).

A significant reduction of Susy mRNA level was observed in S11, S28, and S41 microtubers (Fig. 2A). These selections, Susy cDNA was inserted in a sense orientation for over expression but little Susy mRNA was detected; the enzyme activity was reduced by 5-27% of that of control plants. It seems likely that introduction of the chimeric sense gene into potato plants caused co-suppression of the Susy gene. Suppression of the expression of endogenous genes by introducing homologous sense genes has also been reported for chalcone synthase in petunia (Napoli et al., 1990; Van der Krol et al., 1990), and polygalacturonase in tomato (Smith et al., 1990).

In AS3 and AS10 plants, the strong reduction in the level of Susy mRNA occurred (Fig. 2B), demonstrating that the concentration of endogenous Susy mRNA in microtubers is below the detection level. Concurrently, they also expressed a 63% and 73% reduction, respectively, in Susy activity. These finding are in agreement with the results of Zrenner et al. (1995) who found that a strong reduction in the amount of Susy mRNA is necessary to achieve corresponding decrease in Susy activity. Three lines (8, 22, and 30) of S plants, having higher Susy activity and two lines (3 and 10) of

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Table 1. Analysis of sucrose synthase (Susy) and AGPase activities and starch content of transgenic potato cv. Desiree microtubers.

Plant lines	Susy units · mg protein^{-1} [z]	AGPase nmol glc-1-p · min^{-1} · (mg protein)$^{-1}$	Starch mg · (gFW)$^{-1}$
Control[y]	8.6 ± 1.19	12.8 ± 1.73	184 ± 38.3
TC -1[x]	9.0 ± 1.08	13.1 ± 1.08	200 ± 25.9
S[w] -8	11.9 ± 4.27	15.3 ± 2.51	253 ± 10.2
-22	12.5 ± 2.71	14.5 ± 1.55	345 ± 9.1
-30	12.3 ± 1.97	13.9 ± 2.38	273 ± 11.2
AS[v] -3	3.2 ± 0.01	10.4 ± 1.24	170 ± 17.0
-10	2.9 ± 0.56	9.6 ± 1.19	153 ± 25.4

Data were obtained from five independent plants clonally propagated from an individual transformant. Values are the means ± S. E. between independent microtubers (n=10).

[z] One unit of activity is defined as the amount of enzyme that liberates 1 μmole of fructose per hour.

[y] Non-transformed potato plants.

[x] Transgenic potato plants transformed with pBI-PAT vectors as transgenic control (TC).

[w] Transgenic potato plants with sense (S) gene for Susy.

[v] Transgenic potato plants with antisense (AS) gene for Susy.

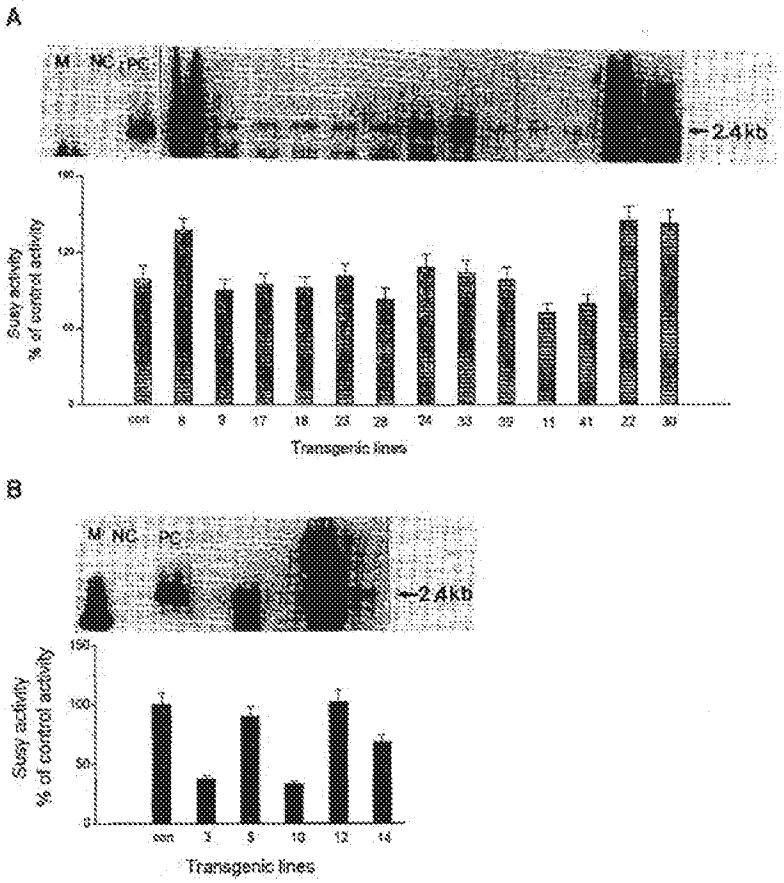


Fig. 2. Gel plates and the relationships between Susy activities and transgenic lines of potato plants (T0) transformed with sense (A) or antisense (B) gene for Susy. DNA fragments (about 10 μl aliquots of PCR reaction), amplified with Susy2 primer and total RNA as a template for 10 cycles, were loaded on 1.0% agarose gel and transferred to a nylon membrane. The blot was UV-crosslinked and hybridized with DIG-labelled DNA probe prepared from pT7BlueSS2. 1kb ladder (M); reaction mixture without template as a negative control (NC); non-transformed control plants (PC); A) S lines, B) AS lines. Susy activity in microtuber extracts is given as a percentage of control plants activity. Data are the means ± S.E. of five different microtubers from each transformant.

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AS plants with lower Susy activity, as compared with control plants, were selected and used for further analysis.

Microtubers with altered Susy activity have increased/reduced starch content

Comparing the influence of altering Susy activity on starch content in microtubers from S (lines 8, 22, and 30), AS (lines 3 and 10) with that of control plants (Table 1) reveals that the starch content in the S plants increased 133-148% over that of the control plants, whereas, in microtubers of the AS plants, the starch content was 83-92% less than that of the control plants. Zrenner et al. (1995) also reported that the starch content of potato tubers, deficient in Susy activity, was reduced by 34-63%.

The microtubers with reduced in starch content had a slightly increased level of sucrose, glucose and fructose (data not shown). However, in the S transgenic microtubers no significant differences were observed in sucrose, glucose, and fructose contents (data not shown). To explain the phenomenon, an exact assay of the reducing sugars will be necessary in the future.

Alteration of the Susy expression altered the accumulation of AGPase transcripts

To test whether the increase/decrease of starch accumulation is the result of altering Susy activity or to the promotion/inhibition of starch biosynthesis, the expression of genes for AGPase was analyzed by RT-PCR. The levels of mRNA for S and B subunits of AGPase were lower in the microtubers of AS 3 plant, and higher in the microtubers of S 8 plants than those of the control plants (Fig. 3) which suggests that some metabolites produced in the Susy sense/antisense microtubers influence the expression of AGPase.

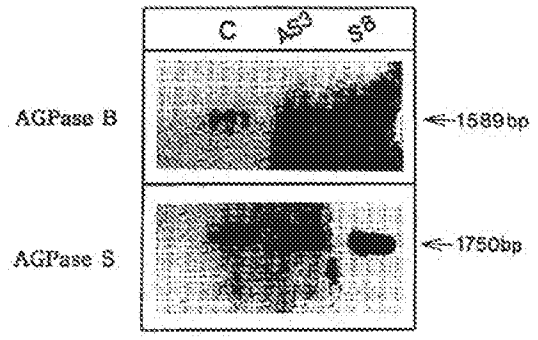


Fig. 3. Gel plates from RT-PCR analyses of AGPase subunit B and S in the control and transgenic S and AS potato plants. DNA fragments (about 10 μl aliquots of PCR reaction), amplified with a set of specific primers for AGPase B or S and total RNA as a template, were loaded on 1.0% agarose gel and transferred to a nylon membrane. The blot was UV-crosslinked and hybridized with DIG-labelled DNA probe prepared from the B22-1 (AGPase B) and S25-1 (AGPase S) cDNAs. C: microtubers from non-transformed control plant.

To investigate whether changes in AGPase mRNA levels reflected the enzyme activity level, the total protein of growing microtubers was extracted and tested for AGPase activity. We found that the specific activity of AGPase in the S transgenic microtubers was slightly higher and that in the AS transgenic ones was lower than those of the controls, although the differences were not significant (Table 1).

These results suggest that the changes in starch accumulation (Table 1) are due to the promotion/inhibition of starch biosynthesis. However, there are many important enzymes that are related to starch biosynthesis, such as UDP-glucose pyrophosphorylase (UGPase), granule-bound starch synthase, and branching enzyme, so that a precise and systematic investigation is required to obtain clear conclusions.

Stability of altered Susy enzyme activity in transgenic plants

Stability of the expression of the transgene in greenhouse-grown plants was further investigated by enzyme and carbohydrates assays. Microtubers of three plants (S8, 22, and 30), overexpressing Susy gene, and two plants (AS 3 and 10), expressing low level of Susy mRNA, were planted and grown under standardized greenhouse conditions. Under these conditions no visible phenotypical differences developed as compared with control plants (data not shown). Five growing tubers, more than 5g fresh weight, per transformant were analyzed for enzyme and carbohydrates assays.

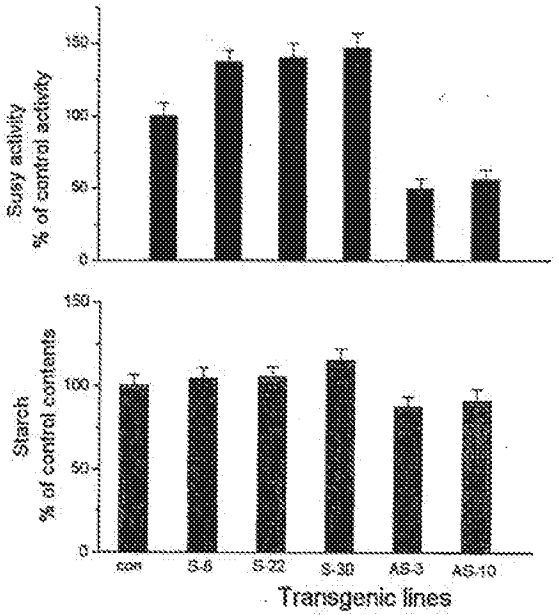


Fig. 4. Relationship between starch contents (upper) and sucrose synthase activity (lower) and transgenic lines of potato tubers grown in a greenhouse. Starch contents and Susy activity in tuber extracts are given as the percentage of those of control tubers. Con: non-transformant. Data are the means ± S.E. of five different tubers.

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Mature tubers of S 8, 22, and 30 plants had higher Susy activity as compared with that of control plants, whereas AS 3 and 10 plants had lower Susy activity (Fig. 4). Starch content of tubers in S plants increased by about 3~5%, while AS plants decreased by about 10%. Thus, the alteration of the Susy gene expression and the resultant change in Susy activity seem to be stable in these three S and two AS plants.

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ジャガイモにおけるスクロース合成酵素遺伝子の導入によるデンプン含量の制御

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摘 要

パタチンプロモーターに，スクロース合成酵素遺伝子の翻訳領域をセンスあるいはアンチセンス方向につないだキメラ遺伝子を，ジャガイモに導入した．形質転換ジャガイモ microtuberのスクロース合成酵素活性とデンプン含量は，センス遺伝子を導入したS8, 22および30の各形質転換体で対照個体に比べて高く，アンチセンス遺伝子を導入したAS3と10では低かった．スクロース合成酵素のmRNAレベルも同様に，これらのセンス遺伝子導入個体では高く，アンチセンス遺伝子導入個体で低かった．デンプン合成系のキー酵素の一つであるADPグルコースピロホスホリラーゼのmRNAレベルと酵素活性をS8とAS3で調べたところ，S8で高くAS3で低かったが，その違いは小さかった．

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ORIGINAL PAPER

Broad spectrum late blight resistance in potato differential set plants Ma*R8* and Ma*R9* is conferred by multiple stacked *R* genes

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Abstract *Phytophthora infestans* is the causal agent of late blight in potato. The Mexican species *Solanum demissum* is well known as a good resistance source. Among the 11 *R* gene differentials, which were introgressed from *S. demissum*, especially *R8* and *R9* differentials showed broad spectrum resistance both under laboratory and under field conditions. In order to gather more information about the resistance of the *R8* and *R9* differentials, F1 and BC1 populations were made by crossing Mastenbroek (Ma) *R8* and *R9* clones to susceptible plants. Parents and offspring plants were examined for their pathogen recognition specificities using agroinfiltration with known *Avr* genes, detached leaf assays (DLA) with selected isolates, and gene-specific markers. An important observation was the discrepancy between DLA and field trial results for *Pi* isolate IPO-C in all F1 and BC1 populations, so therefore also field trial results were included in our characterization. It was shown that in Ma*R8* and Ma*R9*, respectively, at least four (*R3a*, *R3b*, *R4,* and *R8*) and seven (*R1*, *Rpi-abpt1*, *R3a*, *R3b*, *R4*, *R8*, *R9*) *R* genes were present. Analysis of Ma*R8* and Ma*R9* offspring plants, that contained different combinations of multiple resistance genes, showed that *R* gene stacking contributed to the *Pi* recognition spectrum. Also, using a *Pi* virulence monitoring system in the field, it was shown that stacking of multiple *R* genes strongly delayed the onset of late blight symptoms. The contribution of *R8* to this delay was remarkable since a plant that contained only the *R8* resistance gene still conferred a delay similar to plants with multiple resistance genes, like, e.g., cv Sarpo Mira. Using this "de-stacking" approach, many *R* gene combinations can be made and tested in order to select broad spectrum *R* gene stacks that potentially provide enhanced durability for future application in new late blight resistant varieties.

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Introduction

Potato is the world's most important non-cereal food crop (Park et al. 2009; Rauscher et al. 2006). In many regions where potatoes are grown, one of the most notorious and economically damaging pathogens is *Phytophthora*

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infestans (*Pi*), causing late blight (Halterman et al. 2008; Haverkort et al. 2008). The negative economic impact is $3.5 billion annually in developing countries alone (White and Shaw 2010). Fungicides and healthy seed tubers have been used to control potato late blight in developed countries. However, the use of expensive chemical sprays that have unfavorable environmental consequences, and the emergence of new fungicide resistant isolates, have increased the interest and use of host resistance in potato breeding programs (Grunwald et al. 2001; Fry and Goodwin 1997).

So far, over 20 functional late blight *R* genes, including the *Solanum demissum* genes *R1*, *R2, R3a*, and *R3b*, have been cloned (Ballvora et al. 2002; Huang et al. 2005; Lokossou et al. 2009; Li et al. 2011). The cloned genes all belong to the CC-NB-LRR class and, besides *S. demissum,* they were derived from wild *Solanum* species like *S. bulbocastanum* (Song et al. 2003; van der Vossen et al. 2003, 2005), *S. stoloniferum* and *S. papita*, (Vleeshouwers et al. 2008), *S. venturii* and *S. mochiquense* (Pel et al. 2009; Foster et al. 2009).

Solanum demissum, a hexaploid Mexican wild *Solanum* species, is an important source of resistance to late blight. The major resistance (*R*) genes from *S. demissum* show late blight race-specificity, which is associated with a hypersensitive response that is triggered by the cognate avirulence (*Avr*) genes of incompatible isolates of *Pi* (Vleeshouwers et al. 2011). Eleven *R* gene differentials (Black et al. 1953; Malcolmson and Black 1966; Eide et al. 1959) containing *R* genes introgressed into potato from *S. demissum,* have been collected by Mastenbroek (1952) and are referred to as the Mastenbroek differential set: Ma*R1* until Ma*R11*. Seven genes responsible for resistance within this set have been mapped: *R1* on chromosome 5 (Leonards-Schippers et al. 1992), *R2* on chromosome 4 (Li et al. 1998), *R3a, R3b*, *R6*, and *R7* on chromosome 11 (Huang et al. 2005; El-Kharbotly et al. 1996), and *R8* on chromosome IX (Jo et al. 2011). The differential set was originally thought to represent single late blight resistance factors (classified as *R1–R11*). However, *R1* was also found in the Ma*R5*, Ma*R6*, and Ma*R9* differentials (Trognitz and Trognitz 2007). Also the Ma*R3* differential plant contained two *R* genes, *R3a* and *R3b* (Huang et al. 2005).

In the past, *R* genes of Ma*R1*, *-2*, *-3*, *-4*, and *-10* from *S. demissum* have been introgressed into potato cultivars but were rapidly overcome by the *Pi* populations. In order to enhance durability of domesticated late blight resistance, stacking of two *R* genes, was shown to result in a delay in the onset of late blight disease (Tan et al. 2010). *R* gene stacking using conventional- or marker-assisted breeding is a complicated and time-consuming process due to the multitude of genes to be introgressed and due to the simultaneous introgression of undesired traits. Sarpo Mira is one of the few potato cultivars obtained by conventional breeding, which expresses signicant levels of durable late blight resistance. Recently, it was shown that at least five *R* genes are stacked in cv Sarpo Mira (Rietman 2011). Besides traditional and marker-assisted breeding for late blight resistance, transformation of cloned *R* genes into existing potato varieties has gained renewed interest (Haverkort et al. 2008). Recently, it was shown that simultaneous transformation of multiple *R* genes into susceptible cultivars can be used to produce late blight resistant varieties (Zhu et al. 2011; Forch et al. 2010; BASF on line publication 2011).

Remarkably, among the set of 11 potato *R* gene differentials, Ma*R8* and Ma*R9* have shown a broad spectrum resistance in field trials as well as in detached leaf assays (DLA) against several complex isolates of *Pi* (Lehtinen et al. 2008). Lehtinen et al. proposed that this is probably due to a lack of selection pressure because *R9* has never been introduced into commercial cultivars. Zhang and Kim (2007) collected 261 *Pi* isolates during 3 years from Korea and found all races carried multiple virulence genes and showed virulence to the potato differential plants *R1*, *R3*, *R5*, *R6*, *R7*, *R10,* and *R11*, but not on *R8* and *R9*. Also Bisognin et al. (2002) found *Pi* isolates that were virulent on all *R* gene differentials except *R9* in DLA and on both *R8* and *R9* in field trials. More recently, it was shown that virulence towards *R8* and *R9* differential plants is relatively rare in European *Pi* populations (Chmielarz et al. 2010). In this and other studies (Corbiere et al. 2010; White and Shaw 2010) also cv Sarpo Mira, was included and was shown to express similar levels of resistance to late blight as the Ma*R8* and Ma*R9* in multiple years of field trials in Europe and Northern Africa.

Therefore, Ma*R8* and Ma*R9* differentials are expected to harbor important sources of late blight resistance and it is essential to know more about the genetic background of the resistance in these plants. A combination of DLAs with specific isolates, responsiveness to *Avr* genes and *R* gene-specific molecular markers was used to characterize resistance in Ma*R8* and Ma*R9* plants. We found that both Ma*R8* and Ma*R9* contained multiple resistance genes. In order to test whether broad spectrum resistance is a result of *R* gene stacking or a result of any of the individual *R* genes, plants with single *R* genes and plants with combinations of multiple *R* genes have been selected. Using an "on site" *Pi* virulence monitoring system, these plants were tested for their ability to resist high *Pi* infection pressure.

Materials and methods

Plant material

The Mastenbroek differential set Ma*R1, 2, 3, 4, 8,* and *9* (Mastenbroek 1952), cultivars Sarpo Mira, Concurrent,

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Table 1 PCR primers used in this study for *R1*-, *R2*-, *R3a, R3b,* and *R8* diagnostic markers, and for *R2* and *R3a* homolog mining

Name	Sequence	Tm (°C)	Target gene	Product length (kb)	References
76-2SF2	CACTCGTGACATATCCTCACTA	50	*R1*	1.4 (Fig. 1)	Ballvora et al. (2002)
76-2SR	CAACCCTGGCATGCCACG				
R2-F1	GCTCCTGATACGATCCATG	50	*Rpi-abpt*	0.686 (Fig. 1)	This study
R2-R3	ACGGCTTCTTGAATGAA				
SHa-F	ATCGTTGTCATGCTATGAGATTGTT	56	*R3a*	0.982 (Fig. 1)	Huang et al. (2005)
SHa-R	CTTCAAGGTAGTGGGCAGTATGCTT				
CDP3	RRAGATTCAGCCATKGARATTAAGAAA		*R8*	0.500	Jo et al. (2011)
Adaptor pr.	ACTCGATTCTCAACCCGAAAG				
*R3b*F4	GTCGATGAATGCTATGTTTCTCGAGA	55	*R3b*	0.378 (Fig. 1)	Rietman (2011)
*R3b*R5	ACCAGTTTCTTGCAATTCCAGATTG				
R3-Clade1-F	caccATGGAGATTGGSTTARCARTTGGTGGTG	55	*R3*	2.5-4 (Fig. S2)	This study
R3-Clade1-R	AMAKRYATTYCCHATYGATNWBTATGGTGGRA		family		
R3-Clade2-F	caccATGGAKATTGGCTTWGCWGTTGGTGGKGC	55		3.5 (Fig. S2)	This study
R3-Clade2,3-R	AYWGGDATYYCMHATYRMTAWHTATGGTGGRRAT				
R3-Clade3,4-F	caccATGGADATTGGCTTAGCAGTTGGTKGT	55		2.5-4 (Fig. S2)	This study
R3-Clade4-R	TCACAGSYATTYCYCATYGATCTKTATAGTGGAA				
R2F	ATGGCTGATGCCTTTCTATCATTTGC	55	*R2*	2.5 (Fig. S2)	This study
R2R	TCACAACATATAATTCCGCTTC		family		

F forward, *R* reverse relative to ORF orientation

Bintje, and diploid clone RH89-039-16 (RH) were used for crosses and inoculation experiments. Concurrent is a descendent from the *R10* containing cultivar Estima (Vleeshouwers et al. 2000). F_1 individuals (two populations; $n = 100$) derived from the crosses Ma*R8* × Concurrent and Ma*R9* × Concurrent were produced and maintained in vitro at Wageningen UR plant breeding. 2-week-old in vitro propagated plants of both populations were transferred into the greenhouse. 2 to 4 weeks after transfer, DLA or agroinfiltration was performed.

R gene-specific markers

R1- and *R3a*-specific genetic markers were used for validation of the presence or absence of these genes in Ma*R8* and Ma*R9* (Ballvora et al. 2002; Huang et al. 2005). The *R8*-specific marker was described by Jo et al. (2011) and is a cluster directed profiling marker (CDP3). *Rpi-abpt1* (an *R2* ortholog) specific molecular markers were developed based on alignments of *R2*, *Rpi-abpt*, *R2-like* (Lokossou et al. 2009) and nonfunctional homologs (Lokossou 2010). Stretches of inserted/deleted nucleotides were found, and three conserved primer sets were designed to detect *R2*. All three *R2* primer sets worked well with genomic DNA of Ma*R1*, Ma*R2*, Ma*R3*, Ma*R8*, Ma*R9*, cv. Concurrent, and RH89-039-16 (RH). The *R2* primer set that produced the longest polymerase chain reaction (PCR) product was selected (Table 1) and used in this study. Similarly, the *R3b* marker was designed after alignment of the recently cloned

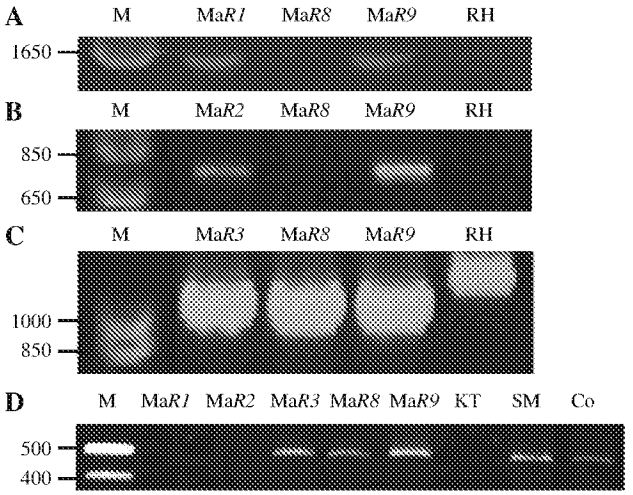


Fig. 1 *R* gene diagnostic markers. PCR products representing **a** *R1*-, **b** *R2*-, **c** *R3a*-, and **d** *R3b*-specific markers in Ma*R1*, Ma*R2*, Ma*R3*, Ma*R8*, and Ma*R9*, RH89-039-16 (RH), Kathadin (KT), Sarpo Mira (SM), and Concurrent (Co). On the left, the size of the DNA marker (M) in bp is provided. The 1,500 bp band produced by the *R3a* marker in RH was an a-specific product

R gene *R3b* (Li et al. 2011) with non-functional homologs. DNA was extracted from plantlets using a high-throughput method (Huang et al. 2005). The PCR primers listed in Table 1 were used in standard PCR reactions with the indicated annealing temperatures. PCR products were separated in a 1% agarose gel and stained with ethidium bromide (Fig. 1).

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Phytophthora infestans assays

The DLA was performed according to Vleeshouwers et al. (2000) using the *Pi* isolates IPO-C, USA618, 99177, 90128, and 99189. These selected isolates were used because of their race specific virulence spectra. The maintenance and preparation of inoculum was according to previously published protocols (Vleeshouwers et al. 2000). Six days after inoculation, resistance versus susceptibility patterns were scored.

"On site" virulence monitoring was performed at three geographically isolated field locations in The Netherlands (Valthermond in the NorthEast, Lelystad in the center and Vredepeel in the SouthEast) in the years 2006–2010. All three locations represent important potato growing areas with high *Pi* infection pressure. Ma*R9*, Ma*R8* and the F1 plants Ma*R8*-18, Ma*R9*-43, Ma*R9*-89, and Ma*R9*-53 were analyzed. Bintje (*R0*), Sarpo Mira and Ma*R1* till Ma*R4,* were used as controls. All plants were propagated from in vitro culture and six plants per genotype were planted at each of the three different locations. This way, the plant material was exposed to the local *Pi* population and weather conditions from beginning of June to mid September without protection by fungicides. Every week, the plants were examined for potato late blight like symptoms. Infected plant material was sampled and used to obtain *Pi* pure cultures. To avoid a local epidemic, dominated by one or a few *Pi* genotypes, all six plants of a genotype were destroyed at the affected location as soon as two pure *Pi* cultures were recovered from this genotype.

The first blight observation date within an experiment was set as day 0. For each genotype, within each experiment and year the first late blight infection was observed and the number of days since the first observation in the field recorded. To estimate the delay in the onset of *Pi* symptoms for the different genotypes, the Censor method was used (Taylor 1973). For statistical analysis, the number of days delay since the start of the epidemic was LOG10(x + 1) transformed. An observation is said to be censored if it is known only that it is less than (or greater than) a particular value. In this case, it is known that some genotypes were not infected during the sampling period. Therefore, the genotype remained free from infection by *Pi* for a fixed number of days (bound value) at a given location in a specific year. Therefore, it is known that the delay at this location and year is longer than the bound value, so this observation is censored. The censored observations were replaced by estimated values for delay, using the method outlined by Taylor (1973).

This method estimates the expected value of each censored observation iteratively conditional on the fact that the value must be greater than the fixed bound, and using the relevant information from the other observations in the experiment. Successively, a T test was performed to estimate significant differences between estimated delays of the different genotypes.

R2 and *R3a* allele mining

R2 allele mining primers were described by Lokossou et al. (2009; Table 1). The high fidelity proofreading DNA polymerase Phusion was used to amplify *R2*-like genes in the following PCR program: 98°C for 30 s. followed by 35 cycles of 98°C for 10 s, 62°C for 10 s and 72°C for 150 s, followed by 72°C for 10 min.

Based on a multiple sequence alignment containing *R3a* homologs from publically accessible databases, a phylogenetic tree was drawn and four clades could be distinguished (Figure S1). Clade-specific degenerate primers were designed and the sequence CACC was added to the 5′ end of the Forward primers to allow directional cloning into D-TOPO vector (Invitrogen, San Diego, CA, USA; Table 1). PCR reactions were done with Phusion™ DNA Polymerase using Ma*R9* and Ma*R8* genomic DNA as template (Figure S2). PCR conditions for *R3* clade one, two, and four specific primers: 30 s at 98°C, followed by 35 cycles of 98°C for 10 s, 65°C for 45 s, 72°C for 180 s, then followed by 72°C for 10 min. Using *R3* clade three specific primer: 30 s at 98°C, followed by 35 cycles of 98°C for 10 s, 62°C for 45 s, 72°C for 180 s; plus a final extension step at 72°C for 10 min.

Both the *R2* and *R3a* PCR products were purified by precipitation using 30% PEG8000/30 mM MgCl2 solution and was inserted into D-TOPO vector (Invitrogen). The recombinant plasmids were transformed into competent cells of *Escherichia coli* strain TOP10 and cultured in Luria-Bertani (LB) broth supplemented with Kanamycin (50 mg/ml). DNA sequencing was conducted on an ABI Prism 3700 DNA sequencer with primer M13F and *R2*- and *R3a*-derived primers inside of inserts.

Sequence similarity was analyzed with Seqman v.7 software (Lasergene, DNAStar, Madison, WI, USA). Sequence comparison was performed with MEGALIGN v.7.

Agroinfiltration assay

Fifteen days after transfer into the greenhouse, F1 population plants derived from Ma*R8* and Ma*R9* and their controls were used for agroinfiltration experiments with *Avr1*, *Avr2*, *Avr3a,* and *Avr4*. Safety regulations for containment determined the use of a separate greenhouse compartment for agro-treatments. 3, 5, and 7 days after agroinfiltration, symptoms which are caused by the interaction between *R* genes and the corresponding *Avr* genes were observed and scored. Agroinfiltration assay followed previously described methodology (Vleeshouwers et al. 2008).

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Table 2 On site *Pi* virulence monitoring

Year	Genotype	*R* gene content	Calculated delay in *Pi* symptoms (days)	Relative delay in *Pi* symtoms*
2006	Bintje	–	4	a . .
	Ma*R1*	1	4	a . .
	Ma*R2*	2	8	a b .
	Ma*R3*	3a, 3b	4	a . .
	Ma*R4*	4	7	a b .
	Ma*R8*	3a, 3b, 4, 8	37	. b .
	Ma*R9*	1, abpt1, 3a, 3b, 4, 8, 9	66	. . c
2007	Bintje	–	1	a . . .
	Ma*R1*	1	2	a b . .
	Ma*R2*	2	2	a b . .
	Ma*R3*	3a, 3b	2	a b . .
	Ma*R4*	4	1	a . . .
	Ma*R8*	3a, 3b, 4, 8	13	. . c d
	Ma*R9*	1, abpt1, 3a, 3b, 4, 8, 9	No lesions**	. . . d
2008	Bintje	–	3	a b . .
	Ma*R1*	1	1	a . . .
	Ma*R2*	2	3	a b . .
	Ma*R3*	3a, 3b	2	a b . .
	Ma*R4*	4	2	a b . .
	Ma*R8*	3a, 3b, 4, 8	24	. . c d
	Ma*R9*	1, abpt1, 3a, 3b, 4, 8, 9	15	. . c .
2009	Bintje	–	1	a . .
	Ma*R8*	3a, 3b, 4, 8	113	. b .
	Ma*R9*	1, abpt1, 3a, 3b, 4, 8, 9	No lesions**	. . c
	Sarpo mira	3a, 3b, 4, smira1, 2	No lesions**	. . c
2010	Bintje	–	3	a . . .
	R8-18	8	50	. b . .
	Ma*R8*	3a, 3b, 4, 8	50	. b . .
	Sarpo mira	3a, 3b, 4, smira1, 2	51	. b . .
	R9-53	3a, 3b, 4, 8, 9	193	. . c .
	Ma*R9*	1, abpt1, 3a, 3b, 4, 8, 9	No lesions**	. . . d

* The onset of *Pi* symptoms was compared between different genotypes using *T* test. Significant differences in delay between different genotypes are represented by different letters. If ranges of letters are overlapping there was no significant difference observed between the genotypes in this particular year

** In case no lesions were found on the tested plants during the course of the experiment, calculations for delay according to censor showed an unrealistic figure, which was replaced by "no lesion"

Results

Low virulence levels in *Pi* populations in the Netherlands towards Ma*R8* and Ma*R9* plants

In order to study whether Ma*R8* and Ma*R9* plants expressed useful resistance to the *Pi* populations in The Netherlands, an "on site virulence monitoring" system was set up. Cv Bintje (R0) was included as reference plant that lacks *Pi* resistance and cv Sarpo Mira was included in the last 2 years of these studies as reference plant with a high level of *Pi* resistance. In addition, four plants from the Mastenbroek differential set were included (Ma*R1*, Ma*R2*, Ma*R3*, Ma*R4*) because their *R* gene content is well described. In all 5 years both Ma*R8* and Ma*R9* performed significantly better than cv Bintje, Ma*R1*, Ma*R2*, Ma*R3*, and Ma*R4* plants, showing that useful resistance is present in Ma*R8* and Ma*R9* plants (Table 2). In 4 of the 5 years, Ma*R9* showed a significantly longer delay in the onset of *Pi* symptoms when compared with Ma*R8*. In 2009 and 2010 cv Sarpo Mira was also included and Ma*R9* and Ma*R8* showed a similar or even longer delay in the onset of *Pi* symptoms. Despite the observed variations per year, it can be concluded that Ma*R8* and Ma*R9* express high levels of resistance to severe *Pi* challenges.

Multiple known *R* genes are stacked in Ma*R8* and Ma*R9* plants

In order to determine the recognition specificity of the resistance in Ma*R8* and Ma*R9*, the plants were screened for their ability to recognize any of the known Avr genes. Using an agroinfiltration assay *Avr1*, *Avr2*, *Avr3a*, and *Avr4* were infiltrated into Ma*R8* and Ma*R9* plants (Fig. 2a;

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Table 3 Avr responses and *R* gene-specific markers in selected Mastenbroek differential set plants, susceptible, and resistant cultivars

	Ma*R1*	Ma*R2*	Ma*R3*	Ma*R4*	Ma*R8*	Ma*R9*	Bintje (*R0*)	Concurrent
Avr1	HR	n	n	n	n	HR	n	n
Avr2	n	HR	n	n	n	HR	n	n
Avr3a	n	n	HR	n	HR	HR	n	n
Avr4	n	n	n	HR	HR	HR	n	n
R3a + Avr3a	HR	HR	HR	HR	HR	HR	HR	HR
pGraB	n	n	n	n	n	n	n	n
R1	+	−	−	−	−	+	−	−
R2	−	+	−	−	−	+	−	−
R3a	−	−	+	−	+	+	−	−
R3b	−	−	+	−	+	+	−	+
R8	−	−	−	−	+	+	−	−

HR hypersensitive response, *n* no hypersensitive response, + presence of specific marker band, − absence of specific marker band

Table 3). Interestingly, Ma*R8* showed an HR reaction with *Avr3a* and *Avr4*. Ma*R9* showed a HR upon infiltration with all tested *Avr* genes; *Avr1*, *Avr2*, *Avr3a,* and *Avr4* (Table 3). These data show that a broad spectrum of *Avr*'s is recognized, which could be explained by the presence of multiple *R* genes or by broad spectrum *R* genes that recognize multiple *Avr*'s.

In order to study whether multiple or single broad spectrum *R* genes were present in the studied plants, Ma*R8* and Ma*R9* were crossed with cv Concurrent. F1 populations were sown and maintained in vitro. Agroinfiltrations of *Avr1*, *Avr2*, *Avr3a,* and *Avr4* were performed in these F1 populations. Segregation patterns were different for each *Avr* (Fig. 2b, Table S1) and it is concluded that different *R* genes must be responsible for their recognition. Because *Avr1*, *2*, *3a,* and *4* are known to be recognized by *R1*, *R2*, *R3a,* and *R4*, our results suggest that *R3a* and *R4* genes are present in Ma*R8* and *R1*, *R2*, *R3a,* and *R4* are present in Ma*R9*. It is, however, an unexpected result since the Ma*R8* and Ma*R9* plants have been selected as *R* gene differentials and were assumed to contain only one *R* gene. In order to confirm the presence of multiple *R* genes in these plants, the molecular basis of the observed effector recognition was investigated.

Solanum demissum late blight *R* genes *R1*, *R2*, and *R3a* have been cloned and their sequences have been used to develop gene-specific molecular markers. The previously published *R1*- and *R3a*-specific markers (Trognitz and Trognitz 2007; Huang et al. 2005) and the *R2*-specific marker (this study) were used to monitor the presence of these genes in Ma*R8*, Ma*R9*, cv Concurrent and RH (Fig. 1), respectively. Also markers derived from the recently cloned *R3b* gene (Li et al. 2011) and the recently mapped *R8* gene (Jo et al. 2011) were included in this study. Ma*R8* was positive for the *R3a, R3b,* and *R8* markers. Ma*R9* showed to be positive for the *R1*, *R2*, *R3a, R3b,*

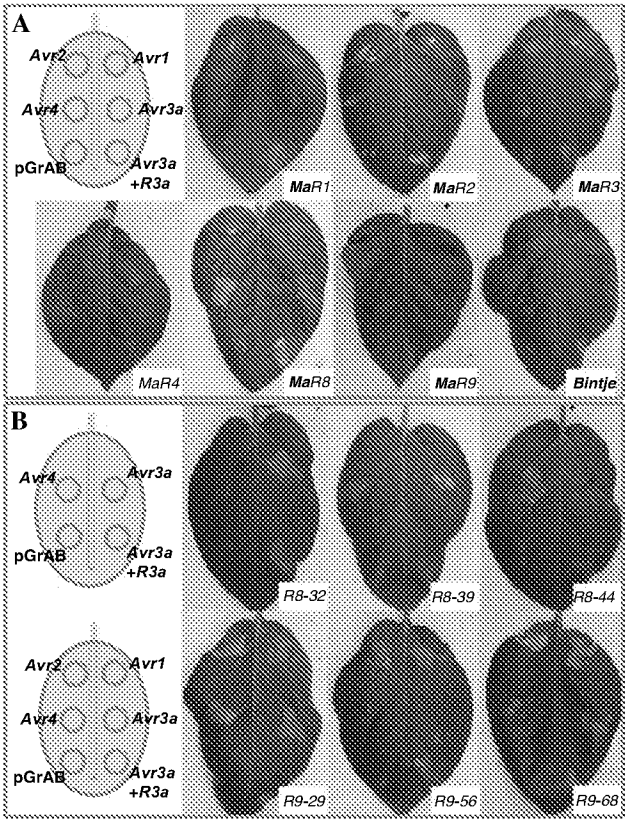


Fig. 2 *Avr1, -2, -3a,* and *-4* induced hypersensitive response. *Avr1, -2, -3a,* and *-4* were agroinfiltrated in Ma*R1*, Ma*R2*, Ma*R3*, Ma*R4*, Ma*R8*, Ma*R9,* and Bintje (**a**) or Ma*R8* and Ma*R9* F1 offspring plants (**a**). pGraB and 1:1 mixture of *R3a* and Avr3a were infiltrated as negative and positive controls, respectively. Representative leaves from three biological replicates are shown

and *R8* markers. Remarkably, also cv Concurrent did show the *R3b* marker band. Cv Concurrent and RH were negative for the *R1*, *R2*, *R3a,* and *R8* markers. Therefore, Ma*R8* is expected to contain *R3a*, *R3b,* and *R8*. Ma*R9* is expected to contain *R1*, *R2*, *R3a, R3b,* and *R8* or closely related homologs

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Table 4 Segregation of *R* gene diagnostic markers, Avr4 responses and disease resistance in F1 and BC1 populations

	F1		BC1	
	3020	3025	3150	3153
	Ma*R8*×C	Ma*R9*×C	*R9*-43×K	*R9*-53×K
R1	Absent	51:49:0		
Rpi-abpt1	Absent	62:38:0		
R3a	49:45:6	88:11:1**		
R3b	62:28:0	88:8:0		
R4 (Avr4)	59:34:7	56:20:24*		
R8	56:44:0	38:51:7**	4:6:0	8:9:0
IPO-C field	55:43:1	95:1:4	4:6:0	12:5:0

Segregation ratios given represent responsive:inresponsive:questionable for Avr4 responses, present:absent:questionable for marker scores, and resistant:susceptible:questionable for disease scores. Data are derived from Table S1

* High number of questionable scores are most likely caused by the strong response to agroinfiltration treatment in this population

** Questionable scores for the *R3a* and *R8* markers were caused by fragments migrating at the same position as the marker bands but had weaker intensities as observed in the other genotypes

thereof. This notion was confirmed upon analysis of the F1 populations. When segregation patterns of *Avr* responsiveness were compared to *R* gene marker segregation patterns a co-segregation was observed (Table S1). Plants that produced a high background cell death response to agroinfiltration and plants that responded poorly to agroinfiltration were excluded from the comparative analyses. *R* gene marker segregation ratios are shown in Table 4. For *R4* no marker was available and here segregation ratios were inferred from effector responsiveness only. It can be concluded that Ma*R8* contains *R3a*, *R3b, R4,* and *R8* in simplex. Overrepresentation of *R3b* in the *R8* population is caused by the presence of *R3b* in the male parent cv Concurrent. In Ma*R9; R1*, *R2, R4,* and *R8* genes seemed to be present in simplex. *R3a* and *R3b* seemed to be present in duplex.

In order to further confirm the presence of the known *R* genes in the plants under study, a *R2* and *R3* (covering both *R3a* and *R3b*) homolog mining approach was pursued. Using primer pairs overlapping the start and stop codons of *R2* and *R3*, dozens of open reading frame (ORF) sequences with high levels of homology to *R2* and *R3,* respectively, were identified in Ma*R8* and Ma*R9* plants. Using the *R2* primers, six clones were identified deriving from Ma*R9*, one clone (9-R-8) was identical at the nucleotide level to *Rpi-abpt1* (Fig. 3). Using the *R3* primers, among 24 clones two exact copies (9-G-52 and 9-G-81) of *R3a* were found in Ma*R9* and no clones identical to *R3b* were found in Ma*R9*. In Ma*R8*, among 6 clones, no exact copies of either *R3a* or *R3b* were found (Fig. 3). The absence of *R3a* in the screening of Ma*R8* and the absence of *R3b* in the screening of both Ma*R8* and Ma*R9* is considered to be due to the low saturation of the screen and does not show that *R3a* or *R3b* are absent in those plants. These results suggest that the *Avr2* and *Avr3a* recognition by the Ma*R9* plants is caused by *Rpi-abpt1* and *R3a,* respectively. Also, because of the sequence identity, it can be ruled out that wider *Avr* spectra are recognized by these individual genes to cause the broad spectrum resistance of the Ma*R9* plants.

R8 and at least one additional *R* gene is present in Ma*R9*

Since it was found that multiple known *R* genes are present in Ma*R8* and Ma*R9*, the possibility of additional, unknown, late blight *R* genes was also investigated. Because of the presence of the multitude of known *R* genes in the studied plants, it was decided to study the populations using a *Pi* isolate, IPO-C, that had overcome *R1*, *R2*, *R3a*, *R3b*, and *R4*, leaving the possibility to study additional resistance genes in Ma*R8* and Ma*R9*. Although the parental plants were resistant to IPO-C in our DLAs, analysis of the F1 populations revealed no (in the case of Ma*R8*) or only very few plants (in the case of the Ma*R9* F1 population) that consistently showed resistance to IPO-C in DLA. However, when the Ma*R8* and Ma*R9* populations were tested in field trials in 2009 and 2010, inoculated with the same isolate (IPO-C), 2 to 4 weeks after inoculation a clear distinction could be made between resistant and susceptible plants. Only a few plants could not be classified as resistant or susceptible and were qualified as questionable (Table 4, Table S1).

It had been described that resistance to IPO-C in the Ma*R8* F1 population, as determined under field conditions, showed a 1:1 segregation and fully co-segregated with the *R8* marker (Jo et al. 2011). The segregation of IPO-C resistance in the Ma*R9* F1 population was severely skewed towards resistance, suggesting that multiple unlinked *R* genes were present. Interestingly, the *R8* marker segregated in a 1:1 fashion (Table 4) in the Ma*R9* F1 population and all the positive plants were resistant to IPO-C. In addition out of 51 plants that lacked the *R8* marker, 46 plants were fully resistant to IPO-C, showing that at least one additional *R* gene is segregating in the population. In order to reduce the *R* gene complexity, BC1 populations were generated deriving from Ma*R9*. Eight resistant F1 plants were selected and crossed with susceptible cultivar Kathadin. The BC1 populations were tested in a field trial inoculated with IPO-C. Two BC1 populations showed a 1:1 segregation for resistance and four populations showed a skewing towards resistance. So, even after crossing to a susceptible plant twice, still a multitude of resistance was observed, deriving from Ma*R9*. The *R8* marker and IPO-C

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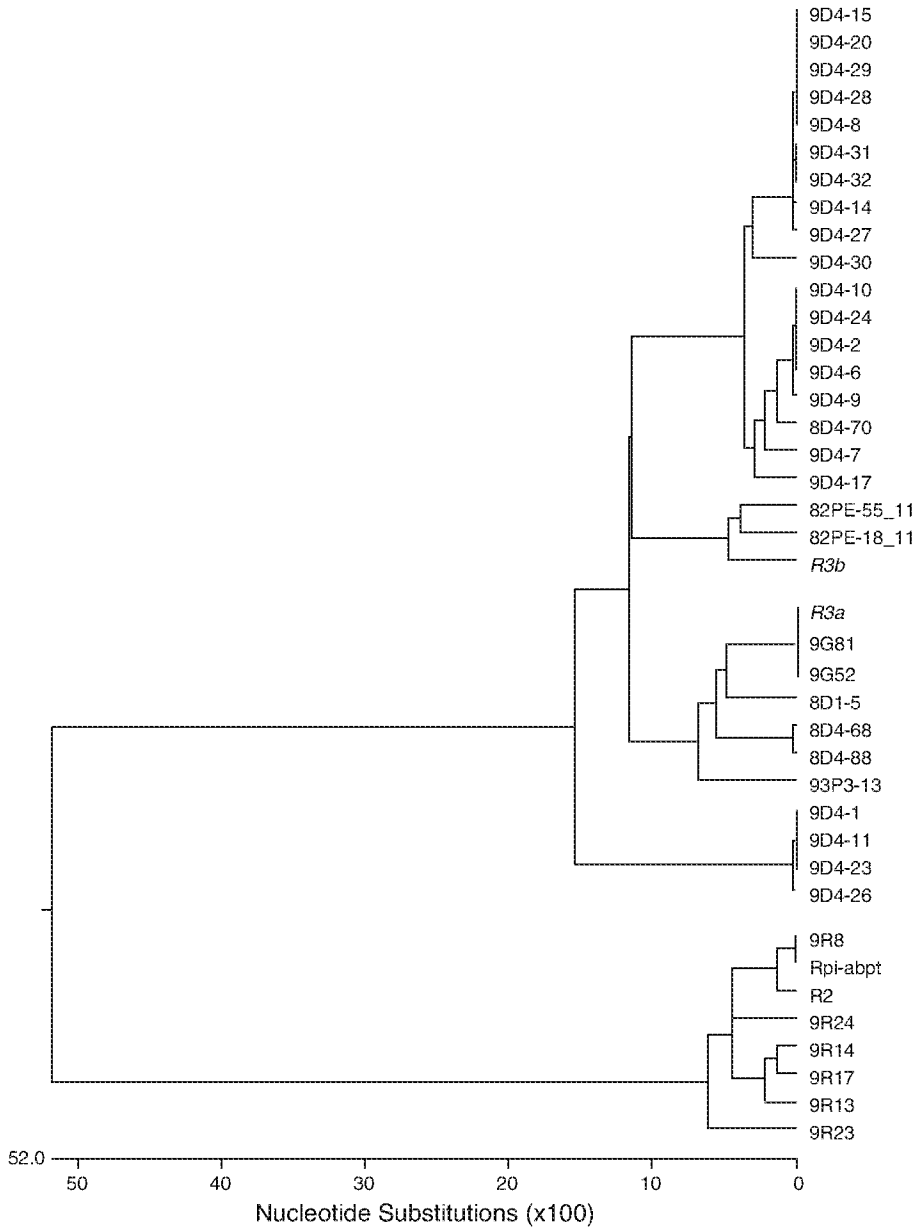


Fig. 3 Phylogenetic analysis of the *R3a* and *R2* homologs. Full-length sequences from allele mining studies with 80% or more nucleotide identity to *R3a* and *R2* were aligned with known *R3a* and *R2* homologs using Megalign from the DNAstar software pack. Sequence names starting with 8 or 9 are derived from Ma*R8* or Ma*R9*, respectively

resistance segregation ratios in two selected BC1 populations (3150 and 3153) are shown in Table 4. A co-segregation from the *R8* marker and IPO-C resistance was observed in population 3150. This showed that the *R8* marker not only tagged a *R* gene from Ma*R8* but also from Ma*R9* and this gene is referred to as *R8* since it is most likely the same gene as the *R8* gene derived from Ma*R8*. In population 3153, that was skewed towards resistance, the *R8* marker was present exclusively in resistant plants, showing that the *R8* gene was also segregating in this population. Also a second *R* gene, deriving from Ma*R9*, was segregating in population 3153 since four plants without the *R8* marker also showed resistance (Table 4). The gene(s) responsible for this resistance is tentatively referred to as *R9*.

Contribution of *R* gene stacking to broad spectrum resistance

The *R* genes within Ma*R8* and Ma*R9* were genetically segregating in different F1 and BC1 populations. This allowed the selection of plants with different combinations of *R* genes that can be used to test for the contribution of each of these *R* genes to *Pi* isolate recognition spectrum. In Table 5, the *R* gene content of selected F1 plants is summarized as derived from *R* gene-specific markers, effector recognition, and IPO-C resistance segregation in field trials.

Five different *Pi* isolates were tested on these plants. As described above and in previous studies, F1 plants containing *R8* and *R9* genes showed no or only weak resistance to IPO-C in DLA. Plant Ma*R9*-71 was an exception. Like the

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Table 5 Resistance spectra of selected differential set plants and of Ma*R8*, Ma*R9* F1 plants

	R gene Content	90128 (Avr2)	99189 (Avr3a, Avr4)	USA618 (Avr4)	99177	IPO-C DLA	IPO-C Field
Ma*R1*	1	S	S	S	S	S	S
Ma*R2*	2	R	S	S	S	S	S
Ma*R3*	3a, 3b	S	R	S	S	S	S
Ma*R4*	4	S	R	R	S	S	S
Ma*R8*	3a, 3b, 4, 8	S	R	R	R	R	R
Ma*R9*	1, 2, 3a, 3b, 4, 8, 9	R	R	R	R	R	R
Concurrent	3b	S	S	S	R	S	S
Bintje	0	S	S	S	S	S	S
F1							
R8-18	8	S	S	S	nd	S	R
R8-69	4,8	S	R	R	nd	S	R
R8-93	3a, 3b, 8	S	R	S	nd	S	R
R9-12	3b, 4, 9	S	R	R	R	S	R
R9-21	2, 3a, 3b, 9	R	R	S	R	S	R
R9-43	3a, 3b, 8	S	R	S	R	q	R
R9-53	2, 3a, 3b, 8, 9	R	R	S	R	q	R
R9-56	1, 2, 9	R	S	S	R	S	R
R9-71	2, 3b, 4, 9	R	R	R	R	R	R
R9-72	2, 3a, 3b, 4, 9	R	R	R	R	S	R
R9-89	3a, 8, 9?	S	R	S	R	S	R

A representative selection of the F1 populations is shown. Resistance to IPO-C was also tested under field conditions. Raw data are presented in Table S1

R resistant, *q* weak resistance or inconsistent results, *S* susceptible, *nd* no data available, *9?* it could not be determined if also *R9* was present in this plant, besides *R8*

parental plant Ma*R9*, this plant did express resistance to IPO-C in DLAs (Table 5). Due to this lack of sufficient biological activity of the *R8* and *R9* genes in DLA, the biological activity of the remaining genes *R2*, *R3a*, *R3b*, and *R4* could be distinguished by the use of differential isolates. None of the selected plants, except *R9*-56, contained *R1* so no discriminating isolate was used for *R1*. Isolate 90128 was avirulent on the plants containing *Rpi-abpt1*. This is in agreement with previous studies and confirms that 90128 expresses *Avr2*. Isolate USA618 was avirulent on all plants containing *R4*, suggesting that this isolate expresses *Avr4*. Isolate 99189 was avirulent on all plants containing *R3a* and *R4*. By comparison to the resistance spectrum of USA618, 99189 could be used as a differential isolate for *R3a*. Isolate 99177 was avirulent on cv Concurrent that was used as the "susceptible" parent for the Ma*R8* and Ma*R9* populations. Resistance to isolate 99177 largely co-segregated with the marker for *R3b* that was derived from parent cv Concurrent. It could, however, not be concluded that isolate 99177 expressed *Avr3b* since it was compatible on Ma*R3* plants that also contained the *R3b* gene. Based on these studies, it can be concluded that stacking of *R2*, *R3a*, *R3b*, and *R4* genes contributes to the isolate resistance spectrum of the plants. It remains, however, to be resolved to what extent stacking of these genes contributes to durability of resistance.

Contribution of *R* gene stacking to durability; a new method to study durability of *R* gene stacks

As described above, the Ma*R8* and Ma*R9* plants showed resistance to high *Pi* infection pressure in "on site" virulence monitoring fields from 2006 to 2010 in three different potato growing areas in the Netherlands. This resistance was similar to the resistance in the durably resistant cultivar Sarpo Mira. In order to set up an *R* gene stack durability assay, the F1 plants from the Ma*R8* and Ma*R9* populations, that contained reduced levels of *R* gene stacking, can be monitored in the same "on site" virulence monitoring fields as the parental plants Ma*R8* and Ma*R9*. In 2010, a first trial was performed (Table 2) and Ma*R8* showed an indistinguishable delay in the onset of late blight symptoms as plant *R8*-18 which contained, besides *R8,* no additional known *R* genes. This suggests that there was no significant

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contribution from the additional *R* genes in Ma*R8*. It must be noted that this is only the first year of these trials. Similar experiments in the years to come must be performed in order to assess whether these findings will show consistency. Remarkable is the finding that cv Sarpo Mira, which contains at least five *R* genes (*R3a*, *R3b*, *R4*, *Rpi-smira1,* and *Rpi-smira2*; Rietman 2011), had a similar delay in the onset of *Pi* symptoms as Ma*R8* and *R8*-18 in 2010. However, in 2009 cv Sarpo Mira remained unaffected while Ma*R8* did show *Pi* symptoms towards the end of the experiment. This showed that differences in virulence levels towards *R* genes in the *Pi* population can vary from year to year. The virtue of *R* gene stacking was illustrated by plant Ma*R9*-53, that besides *R8* also contained *R9*. Ma*R9*-53 showed a significantly prolonged delay in the onset of *Pi* symptoms. The delay in Ma*R9*-53 remained, however, below that of Ma*R9*, which remained unaffected in 2010. This suggests that besides *R8* and *R9* additional factors in Ma*R9* were enhancing the resistance to *Pi*.

Discussion

Molecular basis of resistance in Ma*R8* and Ma*R9*

Ma*R8* and Ma*R9* were exposed in sequential years to high *Pi* infection pressure in trap fields in major potato growing areas in the Netherlands. The onset of late blight symptoms was observed and in every year Ma*R8* and Ma*R9* performed significantly better then control cultivar Bintje and in most cases also significantly better then Ma*R1*, Ma*R2*, Ma*R3,* and Ma*R4* plants (Table 2). In the last 2 years, also comparisons were made to cv Sarpo Mira. In 2009, Ma*R9* showed a similar delay as cv Sarpo Mira, and in 2010 Ma*R8* showed a similar delay as cv Sarpo Mira. In 2010, Ma*R9* even outperformed cv Sarpo Mira. This was in agreement with other comparisons made between Ma*R8*, Ma*R9* and for cv Sarpo Mira in Western Europe and Northern Africa (Chmielarz et al. 2010; Corbiere et al. 2010; White and Shaw 2010). Using a combination of studies including *Avr* responsiveness, *R* gene-specific molecular markers, candidate gene cloning and segregation analysis of these traits in F1 populations, it was possible to show that *R3a*, *R3b*, *R4,* and *R8 S. demissum* late blight resistance genes were present in Ma*R8* and that *R1*, *Rpi-abpt1*, *R3a*, *R3b*, *R4*, *R8, R9* and potentially an additional *R* gene were present in Ma*R9*. This (these) *R9* gene(s) must not be confused with the resistance provided by Ma*R9* and therefore it is only tentatively referred to as *R9*. The additional resistance in Ma*R9* requires further research to identify its molecular and genetic basis. Complicating for this approach will be the difficulty to detect *R9* resistance using DLA in the current F1 and BC1 populations.

R8 and *R9* resistance is background dependent

In F1 plants, a remarkable discrepancy was found between resistance to IPO-C in DLA and in field trials. Parental plants Ma*R8* and Ma*R9* were resistant to IPO-C in DLA and field trials, whereas, only a few F1 plants were consistently resistant to IPO-C in DLA. In field trials, however, at least half of the F1 plants were found to be consistently resistant. Also for cv Sarpo Mira F1 populations, it was reported that DLA resistance to IPO-C was not inherited properly, whereas resistance to IPO-C in field trial conditions was inherited as a dominant trait (Rietman 2011). It has been described before that *R* genes can be poorly segregating traits (Ordonez et al. 1997; Trognitz 1998). Dominant-, resistance-suppressing factors that can be segregating in the same population have been postulated to explain this phenomenon (El-Kharbotly et al 1995). In our populations, such suppressor genes may have derived from the susceptible parent Concurrent. On the other hand, poor resistance segregation may have been caused by multiple accessory genes, deriving from the resistant parent. These accessory genes, required for resistance, may have been present in a heterozygous state and are, therefore, not all present in all offspring plants (Ordonez et al. 1998). Such accessory genes could encode guardees, host components that are guarded (Jones and Dangl 2006) by the R8 or R9 proteins. Alternatively, they could encode downstream signaling components or upstream transcription factors required for the proper transcriptional regulation of the *R8* and *R9* genes. This hypothesis implies that the *R8* and *R9* genes might not be sufficiently active in all genetic backgrounds. Indeed, populations have been found in which the *R8* gene is present and segregating but resistance is only expressed to a very limited extent in field trial conditions (JV, unpublished data). The postulated background dependence of *R8* and *R9* has important implications for GM- and marker-assisted breeding strategies in which multiple broad spectrum *R* genes are stacked. It can be concluded that additional tools need to be developed to determine and distinguish the biological activity of *R* genes in stacks.

Extensive *R* gene stacking in Ma*R8* and Ma*R9* caused broad spectrum resistance

It was shown recently that *R2* and *R3a* homologs could have divergent Avr recognition and, therefore, altered *Pi* recognition spectra (Champouret 2010). This gives rise to the question whether the *R2* and *R3* homologs in the studied plants are responsible for the observed broad spectrum resistance. Cloning of the *R* genes responsible for the recognition of Avr2 and Avr3a from Ma*R9* plants showed sequence identity to *Rpi-abpt1* and *R3a,* respectively. Sequence identity between the genes will result in identical

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proteins and identical recognition specificities, ruling out the possibility that either of these two genes caused the broad spectrum resistance observed in Ma*R9*.

A better explanation for the broad spectrum resistance was found in the *R* gene stacks observed in Ma*R8* and Ma*R9* plants. F1 and BC1 studies revealed the presence of plants with many different combinations of the *R1*, *Rpi-abpt1*, *R3a*, *R3b*, *R4*, *R8,* and *R9* genes. These different combinations resulted in distinct isolate resistance spectra (Table 5). An interesting observation was the resistance spectrum of cv Concurrent, which was used as crossing parent with Ma*R8* and Ma*R9*. This cultivar carries the resistance of Ma*R10* because of its parent cv Estima (Vleeshouwers et al. 2000), which can account for its resistance to isolate 99177. In cv Concurrent, we also found the presence of *R3b*, which locates, like *R10,* to the distal end of the short arm of chromosome 11. It was shown recently that in Ma*R10*, *R3b* and *R10* are two different, closely linked genes (Rietman 2011). Therefore, it cannot be ruled out that plants carrying the *R3b* marker, are in fact containing the *R3b* and *R10* genes, contributing to a different isolate resistance spectrum as plants carrying *R3b* and not *R10*.

Durability in relation to stacking

It is shown that the more *R* genes are present in a plant the broader the isolate resistance spectrum is (Table 5). Theoretically, broad spectrum or multi-*R* gene-based resistance, would result in enhanced durability because it is increasingly less likely that all cognate *Avrs* are lost simultaneously in one *Pi* spore. In the *Pi* population in the Netherlands, however, virulence towards plants containing *R1*, *Rpi-abpt1*, *R3a*, *R3b,* and *R4* is very common (Table 2). When F1 clone *R8*-18, that only contained *R8* and not *R3a*, *R3b,* and *R4*, was analyzed by "on site" virulence monitoring, no difference was observed with the Ma*R8*. Although this is only a 1 year observation, the fact that this was observed at three different locations suggests that the additional *R* genes provided no or minor contribution to the resistance spectrum towards the *Pi* population in the Netherlands. Recent data show that also in cv Sarpo Mira, which has remained durably resistant already for several years, at least five *R* genes are stacked (*R3a*, *R3b*, *R4*, *Rpi-smira1,* and *Rpi-smira2*; Rietman 2011).

The observation that *R8* has a major contribution to resistance in Ma*R8* and Ma*R9* can be explained in two ways. A potential *Avr8* is hard to lose for *Pi* or, the worldwide *Pi* population has not been "trained" yet to evade *R8* recognition because *R8* has not been applied extensively yet in potato varieties. Also a combination of both arguments could hold. In order to avoid *Pi* populations to mutate and evade *R8* recognition, it would be wise to stack additional *R* genes that still show broad spectrum resistance on top of the *R8* gene. The Ma*R9* F1 population described in this study can be used to select for additional *R* genes that combine well with *R8*. Individuals with different known and unknown *R* gene combinations will be selected and will be tested by "on site" virulence monitoring. This will prove an important way to select for *R* gene stacks with potentially improved durability. These stacks could, successively, be selected in marker-assisted breeding programs or could be reconstituted in a GM approach (Jacobsen and Schouten 2007).

Besides cv Sarpo Mira, recently two other potato cultivars have been described to contain durable late blight resistance. C88 is a cultivar that is grown in Asian countries already for more than 10 years and it is now the number one variety in the Chinese province Yunnan due to its late blight resistance (Li et al. 2010). Also the US cultivar Missaukee shows durability already for 7 years (Douches et al. 2010). Of course, durability must prove over the years to come, but also for these two cultivars it would be appropriate to identify the molecular basis of resistance aiding the design of strategies for prolonged durability. Such a strategy could consist of monitoring of virulence within the local *Pi* populations. Virulence towards the *R* genes involved can be detected and can be linked to a fungicide spray advice.

Implications for the *R* gene differential set

A system of tester plants with one *R* gene is essential to determine the virulence spectrum of individual *Pi* isolates. The differential set Ma*R1* till Ma*R11* was long considered to contain one *R* gene per tester plant. However, research during cloning of the *R3* gene clearly indicated that the differential plants from Mastenbroek and Black were different. Ma*R3* contained two resistance genes, *R3a* and *R3b*, and the Bl*R3* differential contained only *R3a* (Huang et al. 2005). Trognitz and Trognitz (2007) showed that the differentials Ma*R5*, Ma*R6,* and Ma*R9* all contain the *R1* gene. The availability of cloned *R* genes and/or matching *Avr* genes enabled to test also the Ma*R8* and Ma*R9* differentials for *R* gene stacking. In this study five *R* gene-specific markers (*R1, R2*, *R3a, R3b,* and *R8*) and four *Avr* genes matching with *R1, R2, R3a,* and *R4* were used. Unexpectedly, three additional *R* genes were found in Ma*R8* and at least five additional *R* genes were found in Ma*R9*. According to our data, race 9 isolates are predicted to also be race 1, 2, 3, 4, 8. Race 8 isolates are predicted to be also race 3, 4. In literature, however, race combinations are found that seem erroneous in retrospect. Consequently, past and future race typing of *Pi* isolates might require careful (re)evaluation using the current knowledge.

Our unpublished data show that also Ma*R5*, Ma*R6*, and Ma*R7* contain multiple *R* genes. Moreover, Rietman (2011)

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showed that Ma*R10* contains *R3b* and *R10*. These findings call for an update of the differential set. An improved or "purified" differential set will allow a more accurate analysis of *Pi* isolate virulence spectra. This could be done by crossing the original differential set plants or their offspring to cultivars without *R* genes. So far, it was possible to select a plant that contained only *R8* (*R8*-18) but a plant with only *R9* remains to be identified. Additional BC2 crosses will be required for this. Such "purified" differential plants will be very useful for future "on site" virulence monitoring. In parallel to an improved differential set, virulence towards *R* genes could be monitored using PCR assays targeting known *Avr* genes. If an *Avr* gene is lost in *Pi* isolates from a geographic location in a potato growing season, this is a good indication that virulence towards the matching *R* gene in the potato population has emerged. Rapid PCR (a)-virulence assays could be linked to fungicide spray advice in a defined geographic region in order to reduce fungicide application dosage and frequency.

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Quantitatively-inherited resistance to *Globodera pallida* is dominated by one major locus in *Solanum spegazzinii*

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Abstract A high level of resistance to *Globodera pallida* pathotypes Pa2 and Pa3 exists in *Solanum spegazzinii*, a wild relative of potato (*S. tuberosum* ssp. *tuberosum*). Here we report the mapping of loci involved in quantitatively-inherited nematode resistance with the use of RFLPs. One major locus, *Gpa*, was mapped on chromosome 5 and two minor loci on chromosomes 4 and 7 of *S. spegazzinii*. Additionally, the contribution of the susceptible parent to nematode resistance was determined. The *Gpa* locus was solely responsible for the high resistance level found in the segregating population. However, the RFLP marker closely linked to this resistance locus showed a distorted segregation, with a shortage of plants having the resistance linked allele. Our results indicate that a prediction of the genetic constitution of a quantitative trait based solely on phenotypic observations can lead to erroneous conclusions.

Key words Potato cyst-nematode · Quantitative trait loci RFLP · Distorted segregation · *Solanum spegazzinii*

Introduction

The potato cyst-nematode *Globodera pallida* (Jones et al. 1970; Stone 1972) can cause severe damage to the potato crop. Three pathotypes of *G. pallida*, Pa1 to Pa3, can be distinguished with a set of differentials which are derived from backcrosses of *Solanum tuberosum* with *S. multidissectum* and with *S. vernei* (Kort et al. 1977). Most cultivated potato varieties (*S. tuberosum* ssp. *tuberosum*) do not posses good resistance to this nematode but resistance against *G. pallida* has been found in wild *Solanum* species. *S. multidissectum* shows resistance to pathotype Pa1 due to the presence of one major gene, H2 (Dunnet 1962). A gene-for-gene relationship between this resistance gene and a *G. pallida* avirulence gene has been described by Parrott (1981). Resistance to pathotypes Pa2 and Pa3 was found in *S. tuberosum* ssp. *andigena* CPC 2802. This resistance was first thought to be monogenic (Howard et al. 1970), but was later proven to be polygenic (Dale and Phillips 1982). Another source of resistance to *G. pallida* is *S. vernei*, which has a quantitative, presumably polygenic, resistance (Ross 1986).

Recently, Arntzen et al. (1993) described a qualitative and monogenic resistance to Pa2 population D236. This resistance was derived from *S. tuberosum* ssp. *andigena* CPC1673. So far, this locus only gives resistance to one population of pathotype Pa2, namely D236, and not to other Pa2 or Pa3 populations tested (Arntzen et al. 1992).

New sources with a broad spectrum resistance to *G. pallida* as well as *G. rostochiensis* have been searched for in other wild *Solanum* species (Van Soest et al. 1983; Dellaert and Hoekstra 1987; Jackson et al. 1988; Turner 1989). Most resistances of these wild species showed a quantitative inheritance, suggesting the action of several genes (Dellaert et al. 1988). *S. spegazzinii* 8218–15 was one of the resistant accessions that had been studied in detail. Dellaert et al. (1988) assumed that the resistance to *G. pallida* pathotype Pa3 was dominated by two to three major genes, each with incomplete resistance and in addition they predicted the presence of minor genes.

Quantitative traits can be dissected into discrete genetic factors with the use of detailed RFLP linkage maps; all regions of the genome can be assayed and accurate estimates of phenotypic effects and genetic positions can be derived (Paterson et al. 1988). In the present paper we describe the identification and mapping of loci, involved in resistance to *G. pallida* pathotypes Pa2 and Pa3, derived from *S. spegazzinii* 8218–15. The aim of this study was to examine

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the assumptions about the genetic basis of resistance made by Dellaert et al. (1988). In addition we investigated the possibility of the presence of QTLs, contributing to the nematode resistance, from the susceptible parent *S. tuberosum*.

Materials and methods

Plant material

S. spegazzinii BGRC accession 8218 seedling number 15 was used as pollinator in a cross with the diploid susceptible *S. tuberosum* SH 78-88-1320. The F1 progeny were tested for resistance to *G. pallida* pathotypes Pa2 and Pa3 (see below). The *S. spegazzinii* parent appeared to be heterozygous at the resistance loci because resistance to pathotypes Pa2 and Pa3 was segregating in the F1 progeny. Mapping of the loci involved in resistance with RFLPs was, therefore, performed in this F1 population.

RFLP analysis

The RFLP markers that were used in this study and the RFLP analysis have both been described earlier by Kreike et al. (1993).

Resistance test

The resistance test for pathotype Pa2 was done in 1988 on 96 F1 plants (total F1 progeny) with nematode population P2-22 (Arntzen and Van Eeuwijk 1992) and for pathotype Pa3 in 1987 (44 plants) and 1989 (39 plants) with nematode population Coll. 1077 (Arntzen and Van Eeuwijk 1992). The F1 population was also tested for resistance against *G. rostochiensis* pathotype Ro1, which is described by Kreike et al. (1993). The resistance tests were performed in five replications in a randomized block design. Thirty cysts enclosed in a nylon net were used as inoculum. After 3 months the newly-formed cysts were collected and counted. A more detailed description of the resistance test is given by Kreike et al. (1993). The differentials VT(N)^{2}62-33-3 and AM78-3778 were used to discriminate between the *G. pallida* pathotypes Pa2 and Pa3 used in this study.

Statistical analysis

A normalizing transformation (^{10}log(x+1)) was performed on the counts of the newly-formed cysts per replicate. These transformed data of the resistance test were investigated with analysis of variance (ANOVA) for a randomized block design. From this ANOVA the heritability of the genotypic mean values for each pathotype was calculated as described by Kreike et al. (1993). The association of marker genotypes with resistance was assessed with one-way ANOVAs based on the averages over the five replicates of the log-transformed cyst counts. In these ANOVAs the data were classified with the alleles of each parent separately i.e., for each marker having two *S. spegazzinii* and two *S. tuberosum* alleles two separate ANOVAs were performed. A significance level of $P< 0.05$ was employed. The magnitude of the marker-associated phenotypic effect is described by the coefficient of determination (R^2), which represents the fraction of the total variance accounted for by the marker genotypes. Since the ANOVA assumes a normal distribution of the data within the classes, and this assumption might be violated, the ANOVA results were verified with a nonparametric test (Mann-Witney U-test).

An RFLP linkage map of chromosome 5 was constructed with the computer program JoinMap (Stam 1993). Interval mapping, as described by Lander and Botstein (1989), could be performed for chromosome 5 for the alleles from the *S. spegazzinii* parent, by treating the F1 as a first generation backcross progeny with fully homozygous genotypes. This analysis was done using the computer program MapQTL, written by Van Ooijen. Readers who are interested in receiving MapQTL should contact Van Ooijen for further details [E-mail (internet): j.w.van.ooijen@cpro.agro.nl].

Results and discussion

Resistance test

The genetic variance in the F1 for both Pa2 and Pa3 resistance was very significant (for both $P< 0.0001$), which means that the different levels of Pa2 and Pa3 resistance found in the F1 population had a genetic basis. The heritability of the genotypic mean values for resistance to *G. pallida* was very high, 0.88 for Pa2 and 0.89 for Pa3 (both 1987 and 1989 experiments). A high correlation (r=0.65) was detected between Pa2 and Pa3 resistance. This indicated either genes with pleiotropic effects, or closely linked genes. No significant correlations (Pa2-Ro1: r=0.17 and Pa3-Ro1: r=0.10) were detected between resistances to *G. pallida* and *G. rostochiensis* (h^2=0.63) (Kreike et. al., 1993) and therefore different, unlinked loci are assumed to be involved in resistance for the two nematode species.

Figure 1 shows the frequency distribution of Pa2 and Pa3 resistance in the F1 population. A continuous range of cyst numbers was found in this population for both pathotypes. Hence, no qualitative inheritance can be detected. The average newly formed number of cysts for the parents, *S. spegazzinii* and *S. tuberosum*, were 1 and 300, respectively. The differential VT(N)^2 62-33-3 was susceptible for Pa3 (on average 128 cysts) while AM78-3778 was resistant for Pa3 (on average 1,2 cysts) but susceptible for Pa2 (on average 63 cysts)

RFLP analysis

One hundred and seven RFLP markers were tested for heterozygosity within the parental genotypes using four restriction enzymes. This search yielded 29 RFLP markers heterozygous for *S. spegazzinii* and 43 RFLP markers for *S. tuberosum*. Table 1 shows the number of heterozygous markers per chromosome for each parent. The segregation of these markers was determined in the F1 population and

Table 1 Number of polymorphic markers used per chromosome to detect association with nematode resistance from *S. spegazzinii* or *S. tuberosum*

Chromosome	*S. spegazzinii*	*S. tuberosum*
1	1	8
2	1	5
3	2	2
4	2	1
5	5	4
6	2	0
7	4	3
8	0	3
9	2	5
10	2	3
11	2	2
12	5	4
Not mapped	1	4
Total	29	43

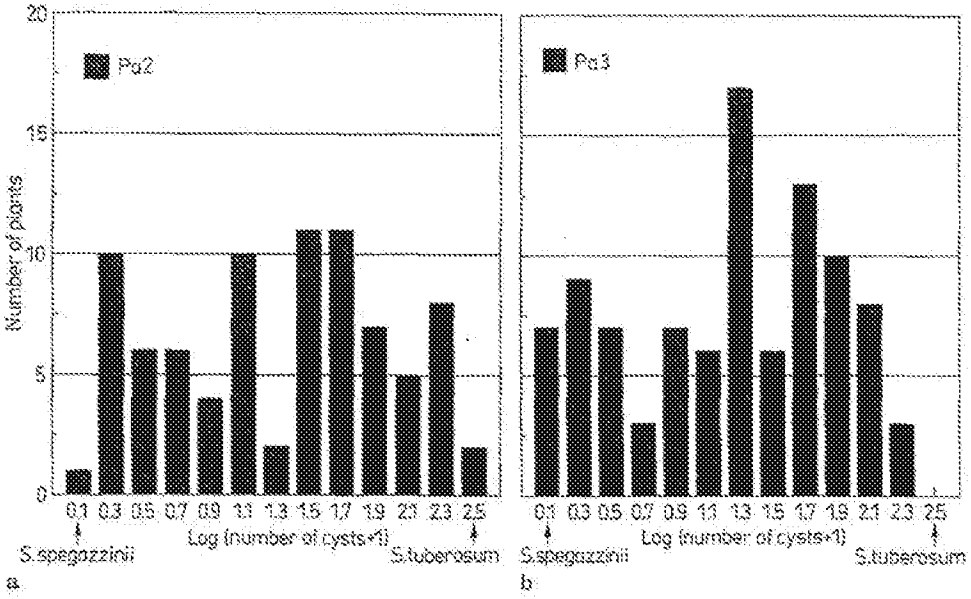


Fig. 1 Frequency distribution of the mean number of cysts found on the F1 plants with Pa2 (a) and Pa3 (b) resistance tests. The data of the resistance tests were transformed [^{10}log(x+1)]

Table 2 Association between RFLP markers and nematode resistance after ANOVA and explained variance per RFLP marker in % of the total phenotypic variance of clone means. *P*-values that are given in parenthesis are considered not significant. Abbreviations: Chr., chromosome; Segr., segregation; Expl. var., explained variance

Marker	Chr.	Segr. ratio	*P* value Pa2	*P* value Pa3	Expl. var. Pa2	Expl. var. Pa3
S. spegazzinii						
Ssp27	4	47:40	(0.50)	0.03	–	5
Ssp124	5	29:22	0.005	0.02	14	9
Ssp112	5	37:30	0.0008	0.002	15	13
Ssp37	5	49:41	0.0001	0.0001	27	22
Ssp72	5	56:24[a]	0.0001	0.0001	51	45
TG143	7	48:33	0.03	0.02	5	7
Ssp51	7	37:39	0.03	0.007	6	9
S. tuberosum						
Ssp56	1	10:32[a]	0.03	(0.11)	9	–
TG130	3	21:33	(0.06)	0.03	–	7
Ssp37	5	34:42	(0.09)	0.03	–	5
Ssp75	11	17:19	(0.22)	0.05	–	9
TG68	12	17:20	0.03	(0.27)	10	–
Total[b]					76	80

[a] Distorted segregation
[b] The highest explained variances for each chromosome are added up to give the total explained variance

used for linkage analysis, which is described by Kreike et al. (1993).

Mapping of the resistance loci

The association of marker genotypes with nematode resistance was assessed with one-way ANOVAs, of which the results are shown in Table 2. Significant associations ($P < 0.05$) indicated that the RFLP marker was linked to a resistance locus. As a verification, associations were also determined with a nonparametric test and these confirmed the results found with the one-way ANOVAs.

The RFLP markers, Ssp124, Ssp112, Ssp37 and Ssp72, with the highest significance for association with nematode resistance were all located on chromosome 5 (Table 2) and showed association with *G. pallida* pathotype Pa2, as well as pathotype Pa3, resistance. Also two markers on chromosome 7, TG143 and Ssp51, showed association with resistance to both pathotypes. On chromosome 4, however, marker Ssp27 was associated only with resistance to *G. pallida* pathotype Pa3.

In order to obtain an estimate of the map position of the resistance locus, interval mapping (Lander and Botstein 1989) was performed for chromosome 5 in which an RFLP map could be constructed. The F1 population was treated as a first generation backcross population by using the two *S. spegazzinii* alleles for each marker only. In this way only the segregation of resistance loci heterozygous in *S. spegazzinii* is studied. The results from the interval mapping on chromosome 5 are presented in Fig. 2a. The threshold was set at LOD 3.0 according to Van Ooijen (1992). These

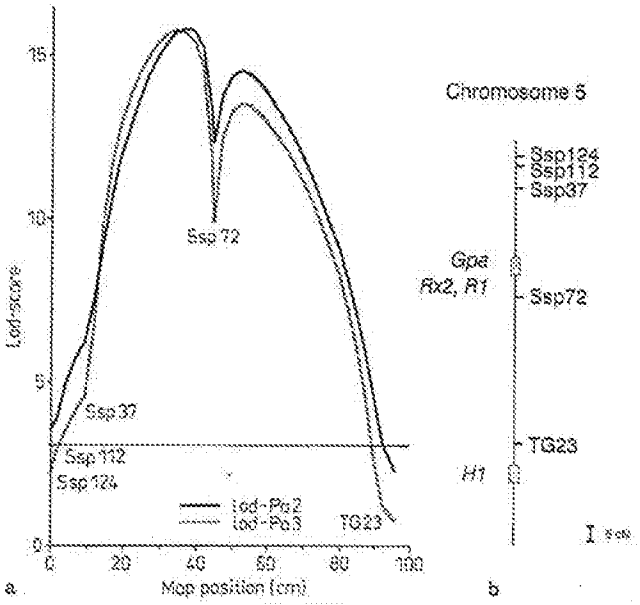


Fig. 2 a LOD scores for *G. pallida* pathotype Pa2 and Pa3 resistance on chromosome 5 of *S. spegazzinii*. **b** Position of the *Gpa* locus on chromosome 5 and the location of other resistance loci on this chromosome (Ritter et al. 1991; Leonards-Schippers et al. 1992; Gebhardt et al. 1993; Pineda et al. 1993)

results show that there is most probably one major resistance locus active against both Pa2 and Pa3, which is located between the markers Ssp37 and Ssp72, approximately 10 cM from Ssp72. We propose to name the major resistance locus on chromosome 5, *Gpa*, since it is involved in resistance to *G. pallida* pathotype Pa2 as well as Pa3 (Fig. 2b).

The existence of loci, heterozygous for alleles involved in nematode resistance, in the susceptible parent *S. tuberosum*, was also determined. Indications for the presence of QTLs were found on chromosomes 1, 3, 5, 11 and 12 (Table 2). Since the segregation of the corresponding RFLP alleles was determined on a limited number of plants only, these associations have to be seen as mere indications for the presence of loci contributing to nematode resistance. The existence of these loci, in the susceptible *S. tuberosum* ssp. *tuberosum* has been described previously. Dale and Phillips (1985) found variation in nematode resistance level within *S. tuberosum* ssp. *tuberosum* genotypes, ranging from fully susceptible to fully resistant.

It is possible that the *Gpa* locus does not consists of one locus that is responsible for resistance against both pathotypes but consists of two closely-linked loci, each conferring resistance to an individual pathotype. Four F1plants were found which were resistant [log (x+1)< 1.0] to one pathotype only and susceptible [log (x+1)>1.0] to the other (Table 3). The presence of the *Gpa* locus can be determined with the RFLP markers Ssp72 and Ssp37, which surround the resistance locus. For two plants the presence of this locus cannot be determined because the RFLP pattern of Ssp72, which is closely linked to the locus, is not known. Based on the RFLP markers, plant SP1 did not possess the

Table 3. Mean cyst numbers of F1 plants which are resistant to only one pathotype and susceptible to the other, and the RFLP data for markers Ssp72 and Ssp37 on chromosome 5. In brackets the mean log (x + 1) transformed cyst numbers are given with the standard error. 1, presence of resistance linked RFLP allele; 0, absence; nd, not determined

Plant	Cysts Pa2	Cysts Pa3	Ssp72	Ssp37
SP17	101.4 (2.01+0.19)	9.0 (1.00+0.19)	nd	0
V28	100.2 (2.01+0.21)	3.5 (0.65+0.21)	nd	1
SP1	5.6 (0.82+0.19)	85.3 (1.94+0.19)	0	0
V223	4.9 (0.77+0.21)	42.9 (1.61+0.21)	1	0

Gpa locus, therefore the high-resistance level to pathotype Pa2 could be caused by the action of several minor loci. Based on marker Ssp72, plant V223 most likely possessed the *Gpa* locus, but has a recombination between marker Ssp72 and Ssp37, for which it has the susceptibility-linked allele. The recombination event could have taken place at the *Gpa* locus, separating the Pa2 and the Pa3 pathotype-specific resistance loci.

A large part of the total phenotypic variance could be explained with the associated RFLP markers (Table 2). Marker Ssp72, which is linked to a major locus, explains 51% and 45% of the total variance of Pa2 and Pa3 resistance, respectively. The other markers on chromosome 5 all showed linkage with the same major resistance locus. The markers on chromosomes 4 and 7 from *S. spegazzinii* and on chromosomes 1, 3, 5, 11 and 12 from *S. tuberosum* explained 10% or less of the total variance and are therefore considered to be minor loci. Because we were not able to employ more segregating markers, we could not investigate whether the low explained variance of some markers was due to either a large distance between marker and resistance locus, or to a small genotypic effect of a closely-linked locus. On the other hand, adding up the explained variance of all significant and unlinked markers leads to a total of 76% and 80% of the explained variance for Pa2 and Pa3 respectively (Table 2). And because the heritability for Pa2 and Pa3 resistance was 0.88 and 0.90, respectively, it seems that most of the genetic variantion can be explained with these markers. Hence, we do not expect much larger genotypic effects of the loci linked to these markers.

Classical segregation analysis versus Genetic analysis with markers

Based on the research of Dellaert et al. (1988) we expected to find two to three major loci which are involved in resistance to *G. pallida*. However only one major locus was detected. There are several reasons for this discrepancy. First, Dellaert et al. performed segregation analysis on 44 plants only which is the population tested against Pa3 in 1987

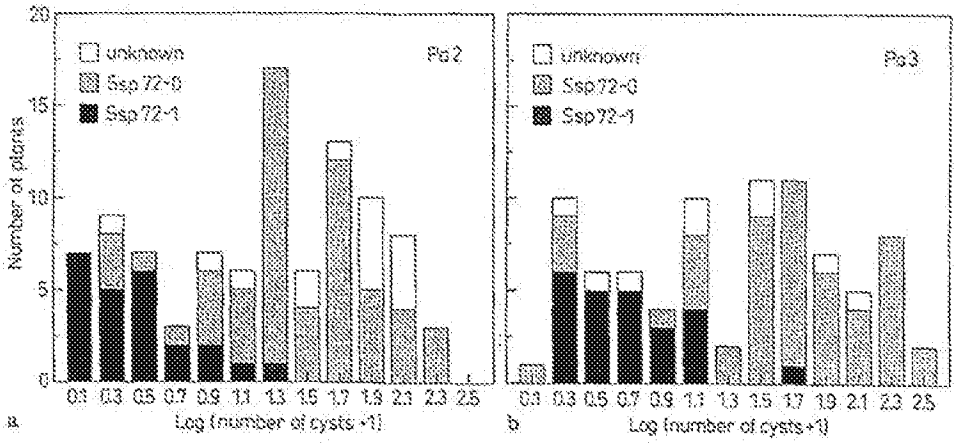


Fig. 3 Presence of the alleles of marker Ssp72, which is closely linked to the *Gpa* locus, in the frequency distribution of Pa2 (a) and Pa3 (b) resistance in the F1 population

also used in the present paper (see Materials and methods). A population size of only 44 plants is relatively small for segregation analysis on a quantitative trait such as the present nematode resistance. Secondly, segregation analysis of a continuously-distributed trait relies heavily on the choice of the threshold for genotype classification (e.g., resistant or susceptible). Genetic analysis based on (molecular) markers circumvents the choice of genotype classification. Thirdly, classical segregation analysis assumes segregation to be according to the Mendelian ratios. The estimation of the number of genes is based on the best fitting segregation ratio, given the chosen-genotype classification and genetic model. Minor deviations are often explained by adjusting the genetic model, for instance, by assuming incomplete dominance or linkage. When, however, genetic analysis with markers is performed, the realised segregation can be determined, and possible distortion can even be tested.

We found that the *Gpa* locus is situated in a region where segregation is distorted. Marker Ssp72, which is closely linked to the resistance locus, showed a skewed segregation with a shortage of the resistance-linked allele (Table 2, Fig. 3). The choice of the genotype classification threshold based on Fig. 1 is quite arbitrary; thus, our results demonstrate that classical segregation analysis can easily lead to erroneous conclusions.

Distorted segregation in this region of potato chromosome 5 has been observed previously (Ritter et al. 1991). Resistance to PVX also showed a segregation ratio that deviated significantly from 1:1 in the F1 progeny. Additionally in this case a marker was found on chromosome 5 which cosegregated with the resistance trait. Several mechanisms that cause distorted segregation are known, such as incompatibility, male /female gamete selection, or even sporophytic selection. The precise mechanism causing this disturbance at chromosome 5 remains to be elucidated.

Other resistance loci on chromosome 5

The *Gpa* locus on chromosome 5 is yet another nematode resistance locus on this chromosome (Fig. 2 b). The H1 gene from *S. tuberosum* ssp. *andigena* CPC 1673 which conferred resistance to *G. rostochiensis* pathotype Ro1 was also mapped on chromosome 5, near TG23 (Gebhardt et al. 1993; Pineda et al. 1993). Furthermore the locus determining resistance to the D236 population of *G. pallida* pathotype Pa2 is probably located on chromosome 5 too, because Arntzen et al. (1993) assumed linkage with the H1 locus. Resistance loci against other pathogens of potato have also been mapped on this chromosome, e.g., the *Rx2* locus against PVX (Ritter et al. 1991), the *R1* locus against *Phytophthora infestans* (Leonards-Schippers et al. 1992), and trichome-mediated insect resistance (Bonierbale et al. 1992)(Fig 2 b). The location of the latter could not be shown in Fig. 2 b, because other markers were used to map this resistance, but its location is most likely in the same region as the *Gpa* locus.

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ORIGINAL PAPER

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Characterization and mapping of *Rpi1*, a late-blight resistance locus from diploid (1EBN) Mexican *Solanum pinnatisectum*

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Abstract *Solanum* is a diverse genus with over 200 species occupying a range of habitats from the Southwestern United States to Central Chile. Germplasm evaluations have focused on species that can be crossed with *S. tuberosum*, while Mexican diploid (2n = 2x = 24) *Solanum* species with an Endosperm Balance Number (EBN) of 1 have received less attention because of poor crossability due to their ploidy and EBN. Recent changes in *Phytophthora infestans* populations have increased the need for new sources of genetic resistance to this fungus. We have characterized resistance to *P. infestans* in the Mexican 2x(1EBN) species *S. pinnatisectum*. An interspecific hybrid between resistant *S. pinnatisectum* and susceptible *S. cardiophyllum* plants was backcrossed to *S. cardiophyllum* to generate a family segregating for late-blight resistance. The diploid (1EBN) genetic map generated with 99 RFLP markers revealed extensive synteny with previously published potato maps. A single dominant late-blight resistance locus (*Rpi1*) from *S. pinnatisectum* was mapped to chromosome 7, a region previously not associated with late-blight resistance. Characterization of the *P.infestans* isolate used for disease evaluations revealed that it possessed the avirulence gene corresponding to the *R9* resistance locus, indicating that *Rpi1* could possibly correspond to *R9*.

Keywords Late blight · *Solanum* · Resistance · 1EBN · Genetic mapping

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Introduction

Late blight of potato (*Solanum tuberosum*) is a devastating fungal disease that attacks the leaves, stems, and tubers of the susceptible plant (Agrios 1988). Over the last 20 years the incidence of late-blight epidemics has increased world wide (Fry et al. 1993). The causative agent is the oomycete fungus *Phytophthora infestans* (Mont.) de Bary, a diploid, heterothallic fungus with two mating types (A1 and A2). Prior to 1980, A1 was the predominant mating type outside of Mexico (Goodwin et al. 1994). Over the following 10–15 years, the A2 mating type appeared throughout the world (Fry et al. 1993; Goodwin et al. 1994). Where both A1 and A2 mating types are present, there is the possibility of sexual recombination and increased genetic diversity among progeny. The oospore represents a possible new source of inoculum because of its capacity for long-term survival under harsh conditions (Drenth et al. 1993). At about the same time as A2 was detected outside of Mexico, metalaxyl resistance became widespread in *P. infestans* populations (Deahl et al. 1993; Goodwin et al. 1996). Metalaxyl was the only curative fungicide available to growers and its loss requires frequent application of protective fungicides during the season to control late blight.

Genetic resistance has long been a favored method of late-blight control. The Mexican hexaploid *S. demissum* has been the most widely used source of resistance to *P. infestans*. Umaerus et al. (1983) refer to crosses between *S. tuberosum* and *S. demissum* carried out by British breeders in the early part of the last century. German breeders utilized hybrids with wild species, most probably *S. demissum*, produced by the U.S. Department of Agriculture and sent to Berlin in 1908 (Reddick 1943; Ross 1966; Umaerus et al. 1983; Wastie et al. 1991). Because of the high levels of resistance and ease of transfer, many cultivars have *S. demissum* in their pedigree. Umaerus et al. (1983) reported that 42 out of 128 German cultivars and 36 out of 76 Dutch cultivars have

R genes that originated from *S. desmissum*. Swiezynski et al. (1991) evaluated seven potato genotypes with *S. demissum* in their pedigree and were able to detect both race-specific resistance and resistance associated with late maturity. To date, 11 race-specific late-blight resistance genes (*R* genes) have been identified as originating from *S. demissum* (Malcolmson and Black 1966; Ewing et al. 2000). Five of these 11 *R* genes have been mapped, *R1* to chromosome 5 (Leonards-Schippers et al. 1992), *R2* to chromosome 4 (Li et al. 1998), and *R3*, *R6*, and *R7* to chromosome 11 (El-Kharbotly et al. 1994, 1996). Overall, *S. demissum* has been credited with contributing large portions of both race-specific and race-nonspecific resistance over the years, and is still considered a valuable source of resistance (Niederhauser and Mills 1953; Umaerus et al. 1983; Colon et al. 1995).

Transfer of desirable traits from wild *Solanum* species to the cultivated potato is frequently prevented due to differences in ploidy and Endosperm Balance Number (EBN). Mexican diploid ($2n = 2x = 24$) species with an EBN of 1 are reproductively isolated from tetraploid *S. tuberosum* (4EBN). For development of viable seed, fertilization must occur so that a 2:1 maternal:paternal EBN is achieved in the central cell/endosperm (Johnston et al. 1980). EBN values, independent of ploidy, allow breeders to predict what crosses are likely to be successful. Therefore, late-blight resistance in 2x(1EBN) species is not readily transferred to 4x(4EBN) cultivars, and characterization of genetic resistance to late blight in 1EBN *Solanum* species has been limited.

Various inventories have reported widespread late-blight resistance among Mexican *Solanum* species. Swiezynski (1990) composed a table of late blight resistance in 34 different *Solanum* species, including five Mexican 2x species. Using both field and laboratory tests, van Soest et al. (1984) evaluated 1962 accessions in the German-Dutch Gene Bank and found the highest levels of late blight resistance in species from the Mexican and Bolivian-Argentinian centers of diversity. Because of the highly complex nature of *P. infestans* populations in Mexico, indigenous species may have developed a high level of race-nonspecific resistance (Reddick 1943; Niederhauser and Mills 1953; Niederhauser et al. 1954). However, other researchers have also suggested that because *Solanum* species in South America have not evolved in the presence of *P. infestans*, their resistance may be race non-specific and more durable (Colon and Budding 1988; Colon et al. 1995). The latter view has undoubtedly been influenced by the predominance of race-specific *R* genes in the Mexican *S. demissum*.

Over the last 15 years at least five genetic maps of diploid *Solanum* species have been developed (Bonierbale et al. 1988; Gebhardt et al. 1989, 1991; Tanksley et al. 1992; Jacobs et al. 1995; Perez et al. 1999). Linkages among RFLP markers revealed relatively few differences between potato and tomato (*Lycopersicon*) genomes, except for paracentric inversions on chromosomes 5, 9, 10, 11, and 12 (Bonierbale et al. 1988; Gebhardt et al. 1991; Tanksley et al. 1992). Comparison of tuber-bearing and non-tuber-bearing *Solanum* species revealed overall synteny; however, multiple translocations were identified on chromosomes 2 and 8 (Perez et al. 1999). Synteny among species in the Solanaceae allow for direct comparative mapping of major traits, such as disease resistance (Grube et al. 2000).

The goal of this study was to characterize and map late-blight resistance from the Mexican 2x(1EBN) species *S. pinnatisectum*. We developed a reliable detached-leaf test for late blight to identify late blight-resistant phenotypes. Mapping of RFLPs allowed direct comparisons of the synteny between this diploid genome and previously published potato and tomato maps. We also characterized the *P. infestans* isolate used in the tests for avirulence genes. Our results establish the basis for the further characterization of late-blight resistance among wild Mexican diploid *Solanum* species.

Materials and methods

Plant material

Late-blight resistance was evaluated on plant introductions (PI) of two Mexican 2x(1EBN) *Solanum* species (Table 1). All plants were from true seed from the NRSP-6 Potato Introduction Station, Sturgeon Bay, Wis.. Late blight differential plants were obtained from Dr. John Helgeson (USDA/ARS, Department of Plant Pathology, University of Wisconsin, Madison, Wis.) and Dr. Gary Secor (Department of Plant Pathology, North Dakota State University, Fargo, N.D.).

Disease evaluation

Plants were grown, on a 16-h photoperiod, from tubers in 15-cm pots. A single fully mature leaf, seven to eight nodes down from the apical meristem on the primary stem, was removed with a razor blade. Three layers of absorbent WypAll paper towels (Kimberly-Clark Cat. No. 05701) were placed in 150-mm plastic petri dishes with a double layer of plastic mesh on top. Then 38 ml of sterile distilled water was added to each plate. Detached leaves were placed on the plastic mesh and then sprayed using a hand-held sprayer, once with sterile distilled water (0.7 ml/plate) and once with a *P. infestans* inoculum (0.7 ml/plate, equivalent to 21,000 spores/plate). Petri dishes were wrapped in parafilm and placed in dark incubators at 18 C with 12-hour light cycle. For all experiments incubator shelves and positions were randomized. Leaves were scored at 6 and 11 days after inoculation, using disease severity indices (DSI) based on the percentage of leaf area affected: 0 = no symptoms, 1 = 0–5%; 3 = 6–25%; 5 = 26–50%; 7 = 51–75%; 9 = 76–100%. All symptoms were positively identified as being due to *P. infestans* based on sporangium morphology, assessed using a stereo microscope. Detached-leaf tests were repeated using the same plants 2 weeks after the first inoculation. Differential plants possessing resistance genes *R0*, *1*, *2*, *3*, *4*, *5*, *6*, *7*, *8*, *9*, *10*, and *11* (Malcolmson and Black 1966) were similarly inoculated and phenotypes scored.

Inoculum was prepared from a single isolate (MSU96) of *P. infestans* US-8 (A2 mating type), provided by Dr. Ken Deahl (USDA/ARS, Beltsville, Md.). MSU96 was also selected for its abundant production of sporangia on Rye A media (Caten and Jinks 1968) and aggressive infection of the susceptible cultivar Green Mountain in detached-leaf tests. After 13 days of growth on Rye A medium at 20 C in the dark, water was added to plates, and sporangia were dislodged using a glass rod and filtered through a

Table 1 Late-blight phenotypes, ploidy, and Endosperm Balance Number (EBN) for plant introductions (PI) of 2x(1EBN) *Solanum* species from Mexico inoculated with isolate MSU96 of *Phytophthora infestans*

Species[a]	PI	Ploidy	EBN[b]	No. of plants tested	Phenotype[c]		
					R	I	S
Solanum cardiophyllum subsp. *cardiophyllum*	279274	2x	1	8	2	2	4
	341232	2x	1	8	6	2	0
	341233	2x	1	8	7	1	0
	341235	2x	1	4	3	1	0
	347759[d]	2x	1	7	0	2	5
	558041	2x	1	8	3	1	4
	595465	2x	1	4	1	1	2
	595468	2x	1	8	2	1	5
Solanum cardiophyllum subsp. *ehrenbergii*	184762	2x	1	8	1	0	7
	184767	2x	1	8	0	0	8
	184771	2x	1	7	0	0	7
	186548	2x	1	7	0	0	7
	251725	2x	1	8	0	0	8
	255519	2x	1	8	0	0	8
	275212	2x	1	6	0	0	6
	275215	2x	1	8	8	0	0
	279272	2x	1	8	1	2	5
	545822	2x	1	2	1	0	1
	545823	2x	1	7	2	1	4
Solanum pinnatisectum	184774	2x	1	8	7	1	0
	186553	2x	1	8	7	1	0
	190115	2x	1	8	6	2	
	230489	2x	1	8	5	3	
	253214[d]	2x	1	8	7	1	
	275230	2x	1	8	4	3	1
	275231	2x	1	8	4	4	
	275234	2x	1	8	7	1	
	275235	2x	1	8	7	1	
	275236	2x	1	8	6	2	
	347766	2x	1	8	8		
	537023	2x	1	8	7	1	
	275233	2x	1	8	7	1	

[a]All species belong to the Series Pinnatisecta according to Hawkes (1990)
[b]Endosperm balance number (Hawkes 1990; Bamberg et al. 1996)
[c]Phenotypes were assessed by measuring mean disease severity indices (see Materials and methods), and were scored as resistant (R) for DSI = 0.0–2.49, intermediate (I) for 2.5–5.9, and susceptible (S) for 6.0–9.0 (6 and 11 day means)
[d]Single plants were selected and used as parents to generate the mapping population

single layer of cheesecloth. Inoculum was incubated at 8–12 C for 2–3 h to induce zoospores. Sporangium counts, using a hemacytometer at 100× magnification with a light microscope, were taken before and after incubation and the average used to adjust concentrations to 30,000 sporangia per ml.

Field evaluations of parental plants and BC1 progenies using natural inoculum were attempted at the Hancock Agricultural Research Station in Central Wisconsin in 1997–1999. Backcross progenies evaluated with detached-leaf tests were also evaluated in the field in 1999 in Hancock, as well as in Toluca, Mexico. However, the late arrival of inoculum at Hancock in 1999, in combination with severe early blight pressure, resulted in insufficient and unreliable late-blight field evaluations. Irregular emergence of plants due to tuber rot and dormancy in Toluca in 1999 confounded attempts to score disease phenotypes there.

Two Mexican 2x(1EBN) *Solanum* species, *S. pinnatisectum* and *S. cardiophyllum* subsp. *cardiophyllum*, were selected based on their ability to produce numerous fertile flowers, adequate tuberization, and viable interspecific hybrids. *S. pinnatisectum* was reported to show resistance to *P. infestans*, while several *S. cardiophyllum* PIs were recorded as being susceptible to the fungus (van Soest et al. 1984; Hermsen 1985; Hanneman and Bamberg 1986). Reciprocal interspecific crosses were conducted between *S. pinnatisectum* PIs 184764, 186553, 186554, 190115, 253214, 275234 and 347766, and the *S. cardiophyllum* subsp. *cardiophyllum* PIs 283062 and 347759. Fertile interspecific hybrids were recovered from the cross between late blight-resistant *S. pinnatisectum* PI 253214 and the susceptible *S. cardiophyllum* PI 347759. A single resistant F_1 individual (2703) was backcrossed to the susceptible *S. cardiophyllum* parent (2JK3A) to generate a mapping population of 115 BC_1 plants.

Crosses

All crosses were conducted under greenhouse conditions using emasculated flowers on whole plants. Natural sunlight was supplemented with light from high-intensity sodium and magnesium lamps to produce a 16 h photoperiod. Fully mature flowers were vibrated to release pollen, which was collected in gelatin capsules, and stored at –20 C over a dessicant (Howard 1978). Fruits were harvested 6 weeks after pollination. Seed was extracted after 2 weeks of storage at room temperature, washed, and dried.

Southern hybridization

All plant tissue was collected from young leaves of greenhouse-grown plants. Protocols for cell lysis, chloroform:isoamyl alcohol extraction and isopropanol precipitation of DNA were as previously described (Kennard et al. 1994). DNA was resuspended in 12.5 ml of 1 M NaCl and equilibrated with 4.25 ml of distilled water and 8.25 ml of Buffer QBT (Qiagen, Valencia, Calif.). DNA was then purified following the Genomic Extraction Protocol for Qiagen Maxi (P-500) columns. Genomic DNA (7.5 µg) was

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digested using 20 U of *Eco*RI (GibcoBRL) or *Hin*dIII (Promega) according to the manufacturer's recommendations. Agarose gels (0.8%) were loaded with the digested DNAs, electrophoresed at 8–10 mA overnight, stained with ethidium bromide, and photographed. Southern transfers to Zetaprobe GT membranes (Bio Rad, Berkeley, Calif.) were as described by Kennard et al. (1994).

Tomato cDNA (CT and CD), tomato genomic (TG), potato genomic (GP), and potato cDNA (CP) probes (134 total) were selected to uniformly cover all twelve chromosomes of the potato genome based on previously published map positions in potato or tomato (Bonierbale et al. 1988; Gebhardt et al. 1989; Tanksley et al. 1992). Additional clones were acquired to increase marker density around previously mapped *R* genes on chromosomes 4, 5, and 11 (Leonards-Schippers et al. 1992; El-Kharbotly et al. 1994, 1996).

Plasmid purification followed standard procedures (Kennard et al. 1994). Insert DNA was recovered by band isolation from 0.8% low-melting-point agarose gels and purified using the QIAEX II Gel Extraction Kit. Approximately 25 ng of insert DNA was radioactively labeled with [P^{32}]dCTP, using the DECAprime II Kit (Ambion, Austin, Tex.). Labeling reactions were passed through Sephadex columns to remove unincorporated label (Sambrook et al. 1989). Southern blots were washed in 0.1×SSC and 0.5% SDS at 65°C for 1 to 2 h. Hybridization solution (6×SSC, 5×Denhardt's solution, 0.5% SDS and 0.1 mg/ml herring sperm DNA) was incubated with blots for 3 h at 65 C, and after adding the labeled probe the filters were incubated overnight at 65°C. Blots were rinsed once with 2×SSC, 0.1% SDS, washed twice with 0.1×SSC, 0.1% SDS at 65°C for 15 min, and finally rinsed with 2×SSC. The blots were then wrapped in plastic film, exposed to XAR-5 X-ray film in X-ray cassettes, and placed at –80°C for 2–7 days. Standard film development procedures were followed (Kodak, Rochester, N.Y.).

Generation of the linkage map

Autoradiograms were scored for segregation of *S. pinnatisectum* bands. Goodness-of-fit to expected segregation ratios was established using Chi-square analyses. Data points were arranged and checked for double crossovers in Map Manager Xp version 0.10 (Manly 1993; Manly and Cudmore 1998). Marker intervals were determined in Map Manager XP at $P \leq 0.001$ and confirmed in Map Maker/Exp version 3.0 (Lander et al. 1987; Lincoln et al. 1993). Linkage order was determined at LOD 3.0 using Mapmaker/Exp version 3.0.

Results

Evaluation for race specificity of MSU96

The presence of previously characterized avirulence (*avr*) genes in isolate MSU96 was evaluated by inoculating a complete set of differential potato plants. All differential plants except *R9* were susceptible to the MSU96 isolate, establishing that MSU96 carries *avr9* (Table 2). Although the *R8* and *R10* differentials had intermediate DSIs of 4.5 ± 3.7 and 5.2 ± 3.0, respectively, at 11 days, they were scored as susceptible to MSU96. Higher standard errors about the mean DSI were observed for inoculations on *R8*, *R9* and *R10* differential plants (Table 2).

Evaluation of *Solanum* species for late-blight resistance

Both resistant and susceptible individuals were found in all tested 2x(1EBN) *Solanum* species from Mexico (Table 1). *S. pinnatisectum* was predominantly resistant (82 of 104 plants) and all of the remaining *S. pinnatisectum* individuals showed intermediate phenotypes. PIs of *S. cardiophyllum* subsp. *ehrenbergii* were mostly composed of susceptible individuals (61 of 77), although all PI 275215 individuals tested were resistant (Table 1). Of the 11 *S. cardiophyllum* subsp. *ehrenbergii* PIs, six producd only susceptible individuals. The *S. cardiophyllum* subsp. *cardiophyllum* material displayed a mixture of resistant (24), susceptible (20), and intermediate (11) phenotypes, except for PI 347759 which was predominantly susceptible.

Table 2 Mean disease severity indices (DSI) at 6 and 11 days after inoculation of late-blight differential plants with isolate MSU96 of *Phytophthora infestans*

Plant[a]	*R* genes	Mean DSI ± SD[b]		Phenotype[c]
LB10	R0	7.8 ± 1.8	9.0 ± 0.0	S
PI 423651	R1	6.8 ± 1.8	9.0 ± 0.0	S
PI 423652	R2	7.8 ± 1.8	9.0 ± 0.0	S
PI 423653	R3	8.6 ± 0.9	9.0 ± 0.0	S
PI 201404	R4	8.6 ± 0.9	9.0 ± 0.0	S
PI 303146	R5	8.2 ± 1.1	9.0 ± 0.0	S
W6	R6	7.0[d]	9.0[d]	S
PI 423655	R7	8.2 ± 1.1	9.0 ± 0.0	S
PI 303149	R8	1.0 ± 1.7	4.5 ± 3.7	S
LB 3	R9	0.1 ± 0.2	2.2 ± 2.2	R
PI 423656	R10	1.0 ± 1.4	5.2 ± 3.0	S
W11	R11	9.0[d]	9.0[d]	S
PI 423657	R1R2	9.0 ± 0.0	9.0 ± 0.0	S
PI 423658	R1R3	6.2 ± 3.3	7.8 ± 2.7	S
PI 201406	R1R4	7.8 ± 1.8	9.0 ± 0.0	S
LB 5	R3R4	7.0 ± 2.8	9.0 ± 0.0	S
PI 215623	R1R2R4	6.2 ± 3.0	7.4 ± 2.6	S
PI 215620	R2R3R4	7.2 ± 2.5	8.2 ± 1.8	S
PI 215618	R1R2R3R4	7.4 ± 0.9	9.0 ± 0.0	S

[a]See Bamberg et al. (1996) and Hanneman and Bamberg (1986)
[b]Mean of five inoculations
[c]Phenotypes scored as resistant (R) for DSI values of 0.0–2.49, intermediate (I) for 2.5–5.9, and susceptible (S) for 6.0–9.0 (6 and 11 day mean)
[d]Inoculations of W6 and W11 were not repeated

Segregation of resistance to *P. infestans*

The cross between the resistant (DSI = 0.8 ± 0.9) *S. pinnatisectum* plant 5JK1D (PI 253214) and the susceptible (DSI = 7.5 ± 2.1) *S. cardiophyllum* subsp. *cardiophyllum* plant 2JK3A (PI 347759) produced four resistant and four susceptible F_1 progenies. All F_1 plants were self incompatible, but were male and female fertile. The resistant F_1 2703 (DSI = 0.8 ± 1.1) was backcrossed as the female to the susceptible parent 2JK3A. The phenotypes of resistant parent 5JK1D and F_1 2703 were not significantly different at 6 and 11 days. Evaluation of 98 BC_1 progenies revealed a bimodal distribution of mean DSI (Fig. 1). Based on this distribution, phenotypes were classified as resistant (DSI less than 4.0) or susceptible (DSI≥4.0) 6 days after inoculation. Progenies with intermediate mean DSI were re-evaluated to confirm phenotypes. BC_1 progenies segregated as 42

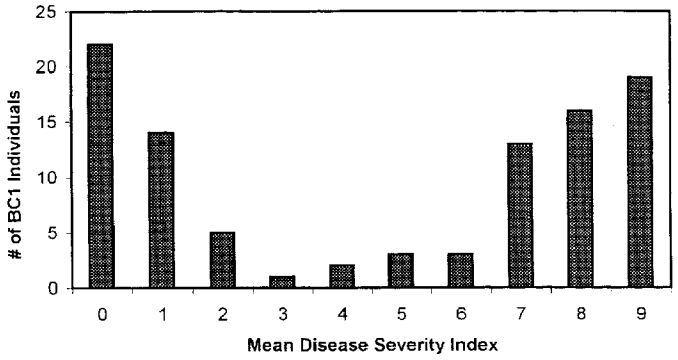


Fig. 1 Numbers of BC_1 progenies that displayed the indicated mean disease severity indices (DSI) 6 days post-inoculation with isolate MSU96 of *Phytophthora infestans*. Values are for three individuals per progeny replicated over two inoculations. DSI values between 0.0 and 3.9 were considered to indicate resistance, and values of 4.0–9.0 susceptibility, as described in Materials and methods

resistant to 56 susceptible, not significantly different from 1:1 (P greater than 0.1). Therefore, resistance to isolate MSU96 was inherited from *S. pinnatisectum* as a dominant allele at a single locus, named *Rpi1*.

Linkage analysis

A total of 134 genomic and cDNA probes were screened for polymorphisms between parental plants using *Eco*RI and *Hin*dIII. Both enzymes revealed the same level of polymorphism (63%). Fifty probes showed polymorphisms with one enzyme, 60 probes with both enzymes. These 110 probes were hybridized to the BC_1 mapping family. A total of 99 probes revealed RFLPs segregating for the *S. pinnatisectum* parental fragments and were used to generate the linkage map. Thirteen linkage groups were resolved at $P=0.001$, for a total of 683.9 cM and an average of eight loci per chromosome (Fig. 2). Two linkage groups seem likely to correspond to chromosome 11. Significant deviations from the

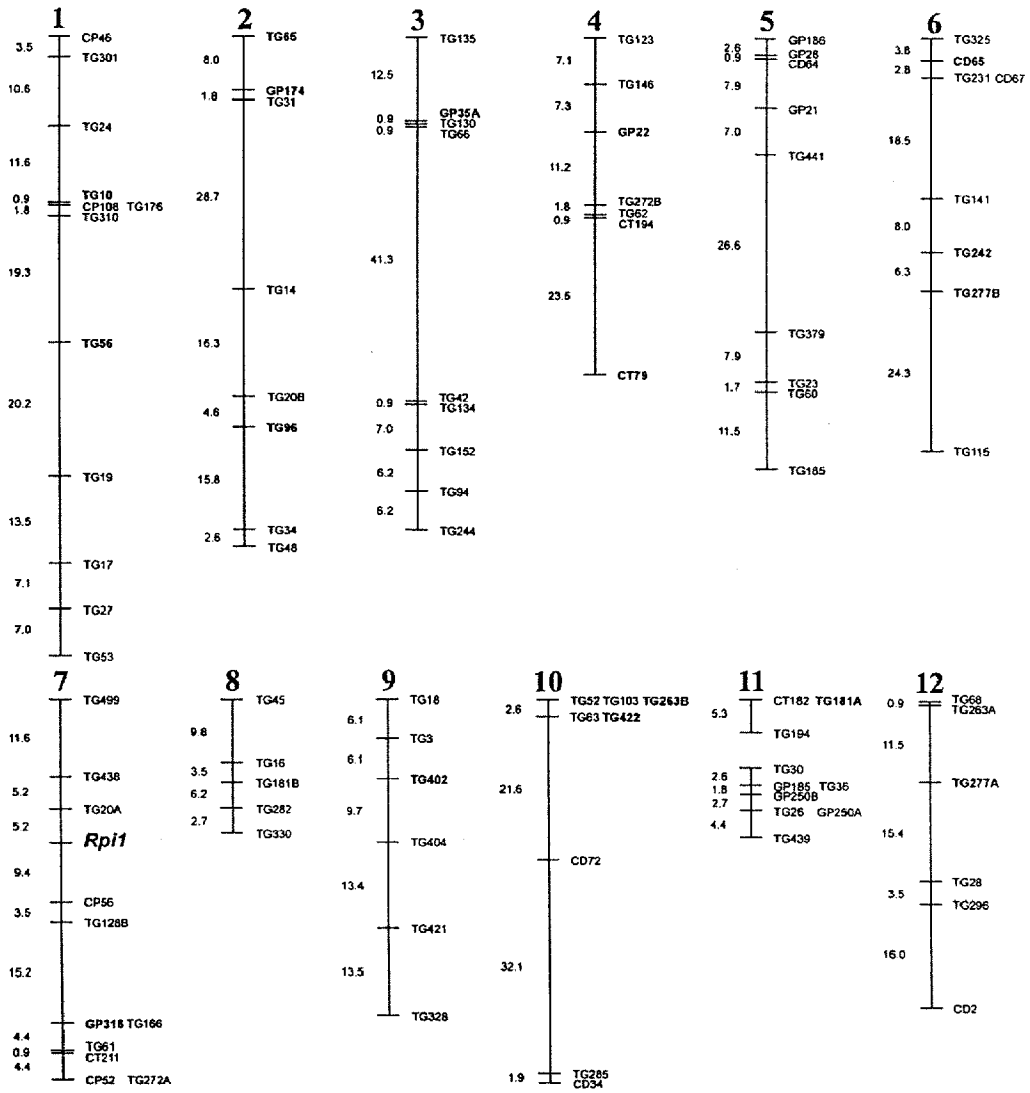


Fig. 2 Linkages among restriction fragment length polymorphisms and late-blight resistance (*Rpi1*) from *S. pinnatisectum* segregating in an interspecific BC_1 population. *Capital letters* at end of probe names indicate that the probes mapped to multiple loci. Probes indicated in *bold* mapped to a different chromosome than in previous maps (Tanksley et al. 1992)

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expected 1:1 ratio were found for 15% of loci ($P \leq 0.01$). Linkage groups 1 and 11a had excesses of *S. cardiophyllum* fragments; while linkage groups 4 and 6 had excesses of heterozygotes with *S. pinnatisectum* fragments. The linear orders of RFLPs corresponded well with previous potato maps (Bonierbale et al. 1988; Gebhardt et al. 1989; Tanksley et al. 1992) (Fig. 3). Eighty-two of the 99 RFLPs revealed synteny with previous potato maps, while seventeen RFLPs, distributed over 10 of the 13 linkage groups, mapped to different linkage groups than reported by Tanksley et al. (1992); however all 17 had complex banding patterns, suggesting gene families with multiple loci.

The late-blight resistance locus from *S. pinnatisectum*, *Rpi1*, was mapped to the central region of chromosome 7 and was flanked by the markers TG20A, *Eco*RI (5.2 cM) and CP56, *Eco*RI (9.4 cM) (Fig. 2).

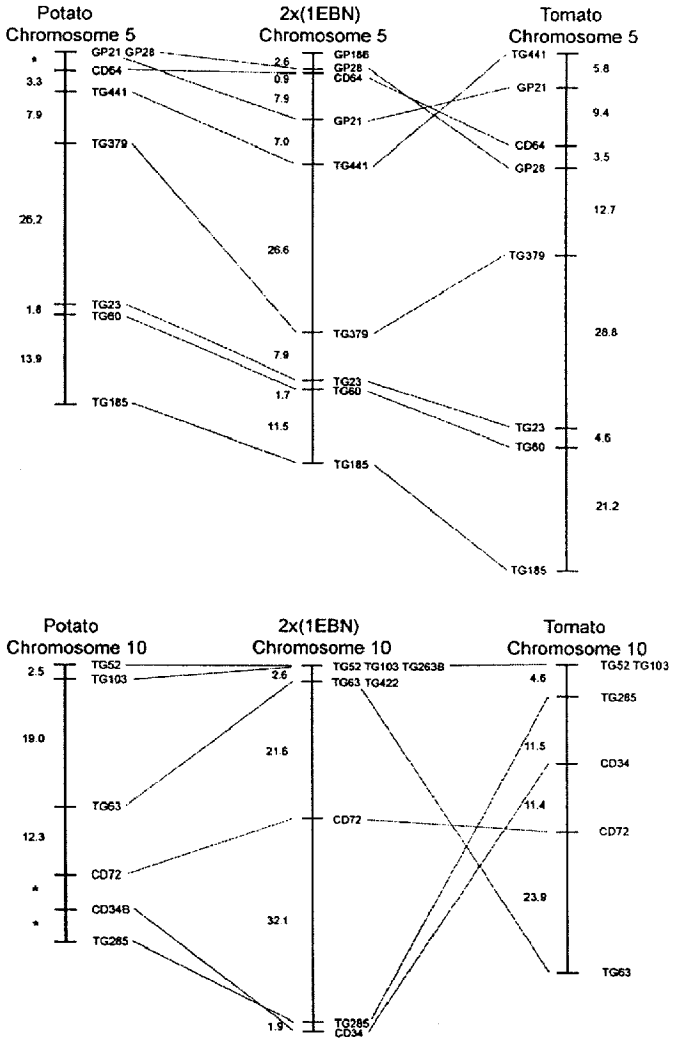


Fig. 3 Synteny between chromosomes 5 and 10 among Mexican 2x(1EBN) *Solanum* species, potato, and tomato (Tanksley et al. 1992). Mexican 2x(1EBN) species show synteny with potato for paracentric inversions on these two chromosomes. The *asterisks* indicate that cM distances and the precise order of markers are unknown (Tanksley et al. 1992)

Discussion

Evaluation of the race specificity of MSU96

Inoculation of the US-8, A2 isolate MSU96 of *P. infestans* onto a complete set of late blight differentials revealed a resistant phenotype on the *R9* differential. Therefore, MSU96 must possess *avr9*. Susceptible phenotypes for all other differentials tested indicated absence or lack of expression of corresponding *avr* alleles. Plants that are resistant to MSU96 may possess *R9*, while all other known *R* genes would not be identified. Therefore, the resistance from *S. pinnatisectum* plant 5JK1D may correspond to *R9*, which originated from *S. demissum* and has not been mapped (Ewing et al. 2000). However, other race-specific genes may be segregating in the F_1 and BC_1 families, but would not be revealed unless the MSU96 isolate possessed the corresponding *avr* gene. Additional experiments are underway to map *29*.

Late-blight resistance in 2x *Solanum* species

Inoculation of *Solanum* species from Mexico with MSU96 revealed a mixture of resistant, intermediate, and susceptible phenotypes. *S. pinnatisectum* was the most resistant Mexican species tested (Table 1). Another Mexican species, *S. cardiophyllum* subsp. *ehrenbergii*, showed 80% susceptibility. As reported by others, Mexican diploid species show a range of resistant and susceptible phenotypes (van Soest et al. 1984).

Segregation of susceptible and resistant F_1 progenies (4:4) from *S. pinnatisectum* × *S. cardiophyllum* indicated that late-blight resistance in *S. pinnatisectum* may be conditioned by a dominant allele at a single locus. The bimodal distribution of late-blight phenotypes in the BC_1 family supports the existence of a qualitatively inherited resistance (Fig. 1). The segregation pattern among BC_1 plants (42:57) supports the idea that a dominant allele at a single locus (*Rpi1*) conditions late-blight resistance in this case. Segregation ratios skewed toward susceptibility have been reported in previous late-blight studies and may be due to specific resistance gene suppressors (El-Kharbotly et al. 1996; Ordoñez et al. 1997). The unreliable nature of the field results from Toluca (Mexico) and Hancock (Wisconsin) prevented confident comparison with our detached-leaf tests.

Synteny of 2x(1EBN) *Solanum* species and cultivated potato

Four previously published genetic maps of potato were generated using mapping populations derived by crossing various tuber-bearing 2x wild species from South America with *S. tuberosum* haploids (Bonierbale et al.

1988; Gebhardt et al. 1989; Tanksley et al. 1992; Jacobs et al. 1995). Perez et al. (1999) constructed a map using non-tuber-bearing 2x South American *Solanum* species. Overall, these maps have revealed synteny among the *Solanum* chromosomes, with the exception of rearrangements on chromosome 2 and 8 of non-tuber-bearing species (Perez et al. 1999). Our mapping between tuber-bearing Mexican diploids, *S. pinnatisectum* and *S. cardiophyllum*, revealed synteny with other tuber-bearing potato genomes (Fig. 3). Tanksley et al. (1992) revealed synteny between potato and tomato, with the exception of five paracentric inversions on chromosomes 5, 9, 10, 11, and 12. The linkage groups within Mexican 2x(1EBN) species aligned well with potato on chromosomes 5 and 10 (Fig. 3); however, too few markers were present on chromosomes 9, 11, and 12 to make confident comparisons. Perez et al. (1999) reported synteny between non-tuber-bearing potato species and tomato on chromosome 10. The overall synteny between our Mexican 2x(1EBN) mapping population and potato supports widespread genome conservation within the Solanaceae and close alignment with other tuber-bearing *Solanum* species.

Mapping of late-blight resistance from *S. pinnatisectum*

Late-blight resistance from *S. pinnatisectum* (*Rpi1*) mapped to chromosome 7. No previously mapped *R* genes are located on this chromosome (Leonards-Schippers et al. 1992; El-Kharbotly at al. 1994, 1996; Li et al. 1998). Synteny of this 2x(1EBN) *Solanum* map with previous potato maps supports the conclusion that *Rpi1* is inherited independently of *R1*, *R2*, *R3*, *R6*, or *R7*. *R9* from *S. demissum* has not been mapped and its relationship to *Rpi1* has yet to be established.

Segregation distortions ($P \leq 0.01$) were observed on four of the 13 linkage groups; only chromosomes 5, 7, and 9 showed no distortions ($P \leq 0.05$). Significant segregation distortions have been observed in numerous mapping studies among other species in the Solanaceae (Bonierbale et al. 1988; Gebhardt et al. 1989, 1991; Chetelat and deVerna 1991, Foolad 1996; Bernacchi and Tanksley 1997). Deviation from the expected Mendelian ratios are commonly observed in segregating families from interspecific crosses (Zamir and Tadmor 1986) and may be due to prezygotic and postzygotic mechanisms, e.g. differential selection during microsporogenesis and/or megasporogenesis, differential fertilization by male gametes, and differential zygote survival (Foolad 1996).

Other disease resistances on chromosome 7

Numerous resistances to bacteria, nematodes, and fungi have been identified on chromosome 7 in potato and tomato (Bournival et al. 1989; Barone et al. 1990; Sarfatti et al. 1991; Kreike et al. 1994; Ballvora et al. 1995; Sandbrink et al. 1995; Leister et al. 1996; Caranta et al. 1997). The resistance loci *I1*, *Gro1*, and *St3.3.2* map within 10 cM of *Rpi1*. In a recent study of disease resistance loci in tomato, potato, and pepper, Grube et al. (2000) identified five intergeneric gene clusters corresponding to regions on tomato chromosomes 3, 4, 9, and 11. However, comparison of gene clusters across genera revealed little correspondence for phenotypically defined genes conferring resistances to related pathogens (Grube et al. 2000). Results reported here support the observation that resistance loci in potato and tomato occur in similar regions of the genome; however, specificity for pathogen recognition is apparently not maintained. Considering the dispersed nature of the loci and the fact that relatively few loci have been mapped, additional corresponding loci with similar pathogen recognition may be expected.

Our characterization and mapping of late blight resistance at the 2x level represents the essential first step towards efficient transfer of late-blight resistance from wild *Solanum* species to the cultivated potato. To transfer late-blight resistance from wild 2x(1EBN) *Solanum* species to cultivated potato, two significant barriers, ploidy and EBN, must be overcome. Both classical and contemporary approaches have proven successful in overcoming these barriers. Chromosome doubling and 2n gametes can be used to manipulate ploidy and EBN for direct crossing (Ehlenfeldt and Hanneman 1984, 1988; Hermsen 1994). Another possible approach is protoplast fusion to directly combine cross-incompatible species (Helgeson et al. 1998).

We now intend to cross the late blight-resistant *S. pinnatisectum* plant 5JK1D, to resistant plants from other 2x(1EBN) species. If *Rpi1* conditions resistance in these other species, we would expect that some interspecific hybrids would be homozygous dominant at *Rpi1*. Test crosses of these plants to susceptible plants would yield only late blight-resistant test cross progenies. However if segregation was always observed in test cross families, additional *R* genes would be identified. These additional sources of resistance will be mapped to identify potentially unique loci for transfer to the cultivated potato.

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ORIGINAL PAPER

Host–pathogen interaction between *Phytophthora infestans* and *Solanum tuberosum* following exposure to short and long daylight hours

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Abstract The influence of short and long day length on the expression of qualitative and quantitative resistance to *Phytophthora infestans* in potato was studied. The incompatible interaction was tested for available set of isolates avirulent in greenhouse conditions to potato Black's differentials possessing the genes: *R*2, *R*5, *R*6, *R*8, *R*9, *R*10, and standard potato cultivar Tarpan (no known *R* gene). The avirulent isolates either were completely avirulent regardless of plant growing conditions, or they infected leaflets of these differentials more frequently when plants were exposed previously to short day conditions than to long day conditions. This study highlights the importance of day length, among many other factors which are controlled, in testing the expression of the virulence of *P. infestans* isolates. In compatible interactions, when quantitative resistance was evaluated in differentials with gene *R*1, *R*3, *R*4, *R*7, *R*11, and potato cultivar Craigs Royal (no known *R* gene), stronger infection expressed by lesion growth rate, as well as stronger sporulation, were observed on potato leaflets of plants exposed to short day for 6–7 weeks before inoculation. The analysis of variance revealed a significant contribution to variation in lesion growth rate of day length, genotype, as well as day length by genotype interaction. Significant influence of isolate, and genotype, but not day length, on the expression of the incubation period was found. The results indicate the necessity of evaluating components of partial resistance present in potato lines used in breeding potato resistant to *P. infestans* in destined day length growing conditions.

Keywords Potato late blight · Avirulence · *R*-genes · Photoperiod

Abbreviations

AULEC	Area under lesion expansion curve
GH	Greenhouse
IE	Infection efficiency
IP	Incubation period
LD	Long day
LGR	Lesion growth rate
MGR	Maximal growth rate
SD	Short day
SI	Sporulation intensity

Introduction

The most economically important potato disease is late blight, caused by *Phytophthora infestans*, which results in 16 % of global yield losses and annual costs of global control and damage in more than €10^9 (Haverkort et al. 2008, 2009), as well as in environmental and economical losses due to chemical control. One of the control measures is to cultivate potato varieties resistant to this pathogen. There are two kinds of resistance expressed in potato plants to *Phytophthora infestans*. Qualitative resistance, conferred by *R*-gene(s), according to the gene-for-gene model (Flor 1971), when effector-triggered immunity is activated, resulting in the hypersensitive response (HR) (Kamoun et al. 1999; Jones and Dangl 2006). This kind of interaction is called incompatible, and the isolate avirulent, when *P. infestans* pathogen secreted RXLR effectors that include

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avirulence (AVR) proteins, are targeted by corresponding resistance (R) proteins from wild *Solanum* species (Vleeshouwers et al. 2011). Race-specific *R*-genes were first found in a wild-potato species *Solanum demissum* Lindl., and then introduced into commercial cultivars (Black et al. 1953; Malcolmson and Black 1966). The resistance was quickly overcome for the first four *S. demissum R*-genes in the 1950s (Howatt and Hodgson 1954). The virulence of *P. infestans* is essential to breeders and plant pathologists willing to use control strategies based on the race-specific *R*-genes (Andrivon et al. 2011). Eleven Black's differentials are applied to assess the virulence of *P. infestans* isolates. Studies on *P. infestans* genome revealed a wide spectrum of fast-evolving effector genes localized to a highly dynamic and expanded region of its genome, and may play a crucial role in the rapid adaptability of the pathogen to host plants (Haas et al. 2009). The assessment of virulence is not easy due to many factors influencing the expression of potato *R*-genes: age of plant, position of tested leaflet, source of inoculum, growing media for pathogen maintenance, spores concentration, and range of environmental conditions, such as humidity, temperature and light intensity (Hodgson and Sharma 1967; Victoria and Thurston 1974; Carnegie and Colhoun 1982; Stewart 1990; Sobkowiak et al. 2004; Rubio-Covarrubias et al. 2005). From our previous observations, the virulence of *P. infestans* isolates assessed on differential plants grown in late autumn was more complex than virulence assessed on plants in summer. It is evaluated in qualitative manner, plants can be either infected or not infected, and the isolate virulent or avirulent, respectively.

The second type of resistance is called as general or quantitative, assumed to be multiple gene-based, and it is evaluated in a quantitative manner, as slower development of the disease resulting in reduced infection efficiency, smaller lesion, lower sporulation and longer latent period (Umaerus 1970; Wastie 1991). The variation of specific components of resistance was revealed among (Colon et al. 1995) and within species (Canizares and Forbes 1995). The importance of infection efficiency (IE), lesion growth rate (LGR) was significant for both *S. tuberosum* and *S. microdontum*, whereas the latent period (LP) was significant for *S. microdontum* and sporulation intensity (SI) for *S. tuberosum*.

The influence of day length on general resistance to *P. infestans* was tested by Mihovilovich et al. (2010). Potato plants were grown in the field in SD or LD conditions, and then evaluated in laboratory assay accordingly in SD or LD conditions. The authors indicated that field resistance to foliar late blight under a given day length depend on the infecting isolate. It was shown that the general resistance expressed as LGR decreased in the short day conditions. The isolate-specific quantitative trait locus that displays interaction with isolate behavior under different day lengths was mapped to chromosome I in the BCT mapping population derived from a backcross of *Solanum berthaultii* to *S. tuberosum*.

To reduce the influence of uncontrolled conditions, we tested the effect of extremely different photoperiod conditions of plant growth on the expression of resistance to *P. infestans*, both the general resistance, after inoculation with three different *P. infestans* isolates, and major gene-dependent resistance after inoculation with a set of 18 isolates. These experiments had following objectives: (i) to test the effect of day length on expression of the *R*-genes in the Black's differentials after inoculation with the set of *P. infestans* isolates, (ii) to evaluate the effect of day length on the expression of general resistance expressed as LGR, IP, and SI to *P. infestans* isolates.

Materials and methods

Plant material

Potato cultivars Craigs Royal and Tarpan, lacking known *R*-gene-mediated resistance to *P. infestans*, the international set of 11 Black's differentials, each possessing a single *R*-gene (*R*1–*R*11) from the Mexican species *Solanum demissum* (Black et al. 1953; Malcolmson and Black 1966), obtained from the in vitro collection at Science and Advice for Scottish Agriculture (SASA), Edinburgh UK) [*R*1, Craigs Snow White; *R*2, 1512(16); *R*3*a*, Pentland Ace (van Poppel et al. 2009); *R*4, 1563c(14); *R*5, 3053-18; *R*6, XD2-21; *R*7, 218ef(7); *R*8, 2424a(5); *R*9, 2573(2); *R*10, 3681ad(1); *R*11, 5008ab(6)].

Phytophthora infestans isolates

The isolates used in these experiments were collected in different locations of Poland from potato to tomato plants in 2004–2007 (Table 1).

Growing conditions of potato plants

Potato plants were produced in the greenhouse from tubers obtained from in vitro plants. 3 weeks after planting, the plants were set into three different conditions. One set of plants remained in a greenhouse (GH), the next two sets were located in two growth chambers with constant temperature 20 °C and different photoperiods: short day (SD) conditions (8 h of light a day), and in long day (LD) conditions (16 h of light a day). The intensity of light was measured at 32 cm of plant height in 10 random places in both chambers with SD and LD light conditions and in the

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Table 1 Data on *Phytophthora infestans* isolates used for inoculation

Isolate	Sample date	Place of origin	The coordinates	Host
MP585	2004.08.17	Kurzętnik	53° 24′N 19° 34′E	Potato
MP674	2005.08.05	Boguchwała	49° 59′N 21° 57′E	Potato
MP750	2006.08.30	Wilków	51° 04′N 15° 58′E	Potato
MP832	2007.08.07	Jęcznik	53° 36′N 20° 53′E	Potato
MP833	2007.08.07	Jęcznik	53° 36′N 20° 53′E	Potato
MP834	2007.08.08	Chomętowo	52° 57′N 15° 39′E	Potato
MP840	2007.08.13	Sulęcin	52° 26′N 15° 06′E	Potato
MP842	2007.08.13	Kalsk	52° 07′N 15° 35′E	Potato
MP846	2007.08.16	Boguchwała	49° 59′N 21° 57′E	Potato
MP847	2007.08.16	Boguchwała	49° 59′N 21° 57′E	Potato
MP851	2007.08.29	Krzydlina Mała	51° 17′N 16° 32′E	Tomato
MP866	2007.07.20	Młochów	52° 03′N 20° 47′E	Tomato
MP868	2007.07.27	Bocheń	52° 06′N 19° 48′E	Tomato
MP870	2007.07.27	Strugienice	52° 07′N 19° 47′E	Potato
MP872	2007.08.06	Glinki	52° 00′N 21° 14′E	Tomato
MP873	2007.08.20	Szewce	50° 38′N 21° 38′E	Tomato
MP879	2007.07.20	Łowicz	52° 06′N 19° 56′E	Tomato
MP880	2007.08.17	Radziejów	52° 37′N 18° 31′E	Potato

greenhouse. Photosynthetic photon flux density (μmol $m^{-2}s^{-1}$) equal 30.7 in SD, 29.7 in LD, 5.28 in GH (measured on a very cloudy day), 69.2 GH (measured on a cloudy day), 403.4 GH (measured on a sunny day). The plants were acclimatized for 5 weeks before the first assessment. The testing was performed in week intervals.

Inoculum preparation

Cultures of isolates were transferred from rye agar onto leaves of cvs Craigs Royal or Tarpan and multiplied for at least two generations, each of 7–10 days. Inoculum consisted of a sporangial suspension that was prepared as described by Zarzycka (2001) from sporulating lesions of potato leaflets and adjusted using a haemocytometer to a concentration of 50,000 sporangia ml^{-1}.

Detached leaflet assay

Replications were leaflets from different fully developed leaves detached from the middle part of the different plants. The number of tested leaflets is listed in Table 2. Leaflets were placed on wet cellulose wadding in a plastic tray. Each leaflet was inoculated by depositing one 30 μl droplet of the inoculum on the abaxial side of the leaf. The trays with leaflets were covered with glass. The inoculated leaflets were incubated for 7 days at 16 °C with a constant illumination of 11.5 μmol $m^{-2}s^{-1}$. After the first 24 h of incubation, the leaflets were turned abaxial side down.

Methods of evaluation

Three experiments were conducted in 2007. Two different methods of evaluation were applied to assess both qualitative and quantitative resistance to *P. infestans*. In 2008, only qualitative resistance was evaluated with the use of avirulent isolates which were available in IHAR collection of *P. infestans* isolates.

Qualitative resistance

The bioassay comprised three experiments in 2007—where each Black's differential, and potato cv. Craigs Royal was tested with three *P. infestans* isolates, and three experiments in 2008—where each of six Black's differentials, and cv. Tarpan, was tested with 15 *P. infestans* isolates (Table 2.). Potato leaflets were sampled from plants grown 5, 6 and 7 weeks under short day length regime (SD), long day length regime (LD), and a greenhouse. The number of tested leaflets from each potato genotype with each *P. infestans* isolate is listed in Table 2. Symptoms were assessed after 7 days of incubation in two grade scale: 0—lack of symptoms or non-sporulating lesions, 1—presence of sporulating lesions.

The phenotype reaction of an isolate with each differential was defined as the pooled result for each set of 12 or 24 leaflets from plants growing in GH conditions. The isolate, which infected less than half of tested leaflets grown in greenhouse conditions, was defined as GH avirulent.

Quantitative resistance

The bioassay comprised two experiments in 2007—six potato genotypes, which showed symptoms of infection, were evaluated: five Black's differentials *R*1, *R*3, *R*4, *R*7, *R*11, and potato cv. Craigs Royal, each with three *P. infestans* isolates. Four leaflets from each plant acclimatized for 6, and 7 weeks in SD or LD conditions were inoculated (Table 2). Symptoms of infection were measured on five consecutive days starting from the second day after inoculation. Resistance was assessed by estimating three components: infection efficiency (IE), lesion growth rate (LGR) and incubation period (IP). LGR was estimated based on the linear regression of mean diameter of lesion as a function of days after inoculation, $y = ax + b$, where y mean diameter of lesion ($\sqrt{\pi cd/4}$), where c and d are vertical and horizontal diameters of lesion, x number of

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Table 2 Scheme of experiments

Type of resistance to *Phytophthora infestans*/evaluation	Years	Tested potato	*Phytophthora infestans* isolate	Number of leaflets tested from each potato genotype with each isolate								
				I[a] (5 weeks)			II[b](6 weeks)			III[c] (7 weeks)		
				SD[d]	LD[e]	GH[f]	SD	LD	GH	SD	LD	GH
Qualitative/symptoms scored on the 7th day after inoculation	2007	*R*1–*R*11, Craigs Royal	MP585, MP674, MP750	4	4	4	4	4	4	4	4	4
	2008	*R*2, *R*5, *R*6, *R*8, *R*9, *R*10, Tarpan	MP832–MP880 [g] (15 isolates)	8	8	8	8	8	8	8	8	8
Quantitative/infection efficiency, lesion growth rate, incubation period	2007	*R*1, *R*3, *R*4, *R*7, *R*11, Craigs Royal	MP585, MP674, MP750	–	–	–	4	4	–	4	4	–
Quantitative/sporulation intensity	2007	*R*1–*R*11, Craigs Royal	MP585	–	–	–	–	–	–	2	2	–

[a,b,c] Tested leaflets detached from potato plants acclimatized in different day length for 5, 6, 7 weeks, respectively

[d,e,f] Plants grown in short day, long day, and in a greenhouse, respectively

[g] *P. infestans* isolates listed in Table 1

days after inoculation, a = LGR expressed as mean increase of the lesion size in mm day^{-1}. Incubation period (IP), the time after inoculation until initial necroses, was defined as $-b\,a^{-1}$. IE expressed as percentage of successful infection. LGR values and IP obtained from the four leaflets of 2 day lengths growing conditions, and of three isolates of *P. infestans*, were subjected to two three factor analysis of variance (ANOVA) using STATISTICA for Windows, Stat Soft, Inc. Tulsa, OK, USA (1997). Variance components were estimated from expected mean squares.

Sporulation intensity (SI) was estimated for all Black's differentials and cv. Craigs Royal after 7 weeks of acclimatization in SD and LD conditions, with the use of the one *P. infestans* isolate—MP585. The area of the infected tissue having an elliptical shape was calculated according to formula $\pi ab/4$ (where a and b are vertical and horizontal diameters of lesion measured on the 6th day after inoculation). Two leaflets per genotype were shaken in 20 ml of water and SI was estimated as a number of sporangia cm^{-2}, based on three to four microscopic counting using a haemocytometer.

Results

Qualitative resistance

Three *P. infestans* isolates, MP585, MP674 and MP750, were virulent to the differentials *R*1, *R*3, *R*4, *R*7, *R*11, and potato cv. Craigs Royal, independent on the growing conditions, in all the three experiments in 2007. Thus, the results for these genotypes are not shown in Table 3.

The *R*5 plants grown in SD, LD, and GH conditions did not show any symptoms of infection after inoculation with 6 *P. infestans* isolates. After inoculation, with other 10 avirulent *P. infestans* isolates, there were observed symptoms of infection more frequently on leaflets originating from SD conditions (69.0 % of leaflets, out of 228 tested, were infected), than from LD, and GH conditions (22.4 and 2.6 % of the leaflets were infected, respectively). This analysis does not include isolates which were not tested in GH conditions. Only in one case, when the *R*5 differential was inoculated with the isolate MP750 different pattern was observed, when 8 leaflets out of 20 from LD conditions were infected, and only 1 leaflet from SD conditions. After inoculation with the isolate MP832 symptoms were observed on leaves from both SD and LD, but not on leaflets from greenhouse plants (Table 3).

No differences in infection were observed for 6 isolates of *P. infestans* and did not infect any leaflets of *R*6. Another 9 avirulent isolates infected, 45.6 % of leaflets from SD (out of 204 leaflets tested), 4.4 % from LD, and 0.1 % from GH conditions.

Ten isolates were avirulent to differential *R*8. Symptoms of infection were observed on 58.3 % of leaflets from SD conditions, 31.4 % from LD conditions and 10.3 % from GH (204 leaflets were inoculated).

Among 18 GH avirulent isolates to differential *R*9, 13 did not infect any leaflets from SD to LD conditions. Out of the 96 leaflets inoculated with 5 isolates, 38.5 % of infected leaflets were grown in SD, 4.2 % in LD, 0.0 % in GH.

Six GH avirulent isolates to *R*10 showed infection of 20.8, 13.2, and 0.0 % of leaflets in SD, LD, and GH,

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Table 3 Number of infected leaflets with 18 *Phytophthora infestans* isolates of Black's differentials *R*5, *R*6, *R*8, *R*9, *R*10, grown for 3–7 weeks in short (SD), long day (LD), or greenhouse (GH) conditions

Isolate	No of leaflets tested per each combination	Number of infected leaflets														
		*R*5			*R*6			*R*8			*R*9			*R*10		
		SD	LD	GH	SD	LD	GH	SD	LD	GH	SD	LD	GH	SD	LD	GH
MP832	24	24	20	0	24	24	23	23	8	4	20	1	0	24	23	24
MP846	24	22	4	2	2	0	0	22	13	13	0	0	0	24	22	22
MP847	24	22	1	0	16	1	0	24	22	16	0	0	0	24	24	24
MP834	24	19	8	3	4	1	2	9	6	4	0	0	0	24	17	12
MP840	24	19	3	0	13	0	0	21	20	12	0	0	0	23	20	20
MP880	24	17	7	1	11	2	0	24	21	19	0	0	0	24	24	24
MP674	12	12	5	nt	12	12	12	1	0	0	3	0	0	12	9	nt
MP585	12	11	4	nt	12	12	12	5	4	0	12	0	0	12	3	nt
MP842	24	15	4	0	13	2	0	24	24	7	0	0	0	24	24	24
MP833	24	14	0	0	15	0	0	24	17	12	0	0	0	24	24	24
MP851	24	4	0	0	10	3	0	24	10	23	0	0	0	24	16	14
MP750	12	1	4	0	9	0	0	12	5	0	0	0	0	12	6	nt
MP866	24	0	0	0	0	0	0	6	4	2	0	0	0	2	0	0
MP868	24	0	0	0	0	0	0	6	3	0	0	0	0	8	6	0
MP870	24	0	0	0	0	0	0	15	5	0	0	0	0	1	0	0
MP872	24	0	0	0	0	0	0	21	17	17	2	0	0	15	11	0
MP873	24	0	0	0	0	0	0	18	5	4	0	3	0	3	2	0
MP879	24	0	0	0	0	0	0	12	12	15	0	0	0	1	0	0
Sum	396	180	60	6	141	57	49	291	196	148	37	4	0	281	231	188

nt not tested

respectively (144 leaflets tested). Different reaction of R9 and R10 differentials is shown in Fig. 1.

The influence of day length was not observed for the *R*2 plants inoculated with *P. infestans* isolates. The leaflets detached from these plants showed symptoms of infection after inoculation with three virulent isolates and did not show any symptoms of infection after inoculation with 15 avirulent isolates of *P. infestans*.

Quantitative resistance

All virulent isolates infected all inoculated leaflets. IE was equal to 100 % in both SD and LD conditions; that is why, this component was excluded from the analysis. LGR and IP were used for analysis of variance. A significant influence of the growing conditions (SD and LD) explained 93.6 % of total variation. In addition, genotype and genotype by growing conditions interaction, on expression of LGR was revealed (Table 4.). The effect of isolate and the interaction of growing conditions with isolate on the LGR was not found. In general, LGR was higher for plants grown in SD than in LD conditions, the mean of LGR was equal to 9.13 and 5.93 mm day^{-1}, respectively. The analysis of variance for IP revealed statistically significant contribution of isolate (31.7 % of total variation), and of genotype (26.0 % of total variation) on this trait, but not of growing conditions (Table 4).

Tested genotypes differed from each other for LGR, and also different reaction of genotypes was observed depending on growing conditions (Table 5). The correlation coefficient between LGR of plants grown in SD and LD conditions was not significant and equal $r = 0.235$ ($P = 0.654$).

Out of the three isolates tested, the isolate MP674 was significantly faster in infection, which was expressed by shortest IP equal 2.14 days, when for MP585 and MP750 equal 2.25 and 2.29, respectively.

The significant differences were observed for SI for plants grown in different photoperiods. Higher SI was observed on leaflets detached from plants grown in SD conditions for cv. Craigs Royal, *R*2, *R*4, *R*6 and *R*7. For the differentials *R*5, *R*9, *R*10 and *R*11, sporulation was observed only on leaflets grown in SD conditions. No significant differences in SI were observed on both differentials *R*1 and *R*3 grown in SD and LD conditions (Fig. 2).

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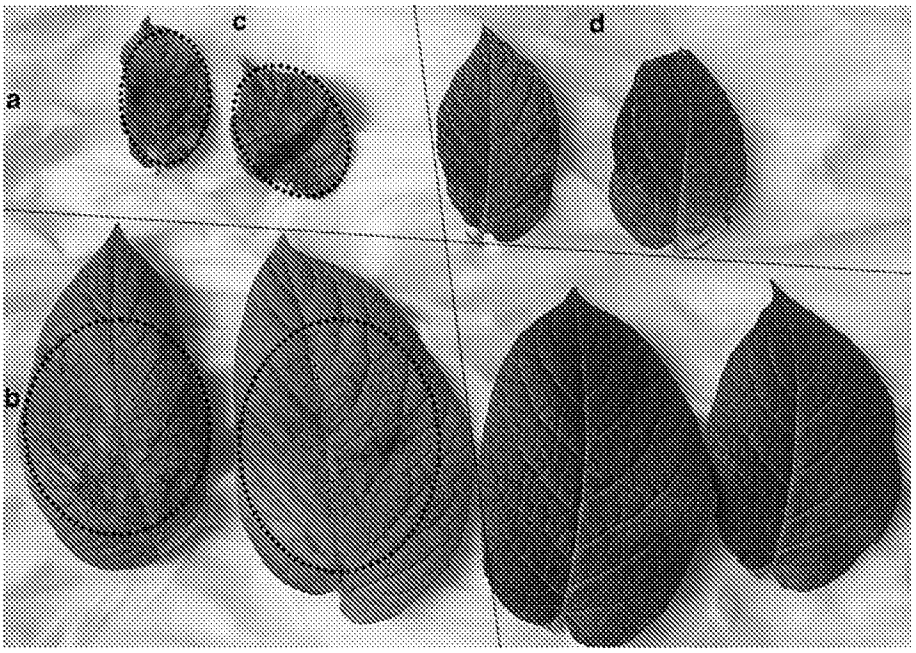


Fig. 1 Bioassays demonstrate the different recognition of the isolate MP585 by the Black's differential *R*9 and *R*10 on the 6th day post-inoculation: **a** the *R*9 differential, **b** the *R*10 differential, **c** leaflets from plants grown in short day conditions (spreading sporulating lesions), **d** leaflets from plants grown in long day conditions (resistant, lack of infection)

Table 4 Analysis of variance on the lesion growth rate (LGR) and incubation period (IP) of potato leaflets of Black's differentials *R*1, *R*3, *R*4, *R*7, *R*11, and potato cultivar Craigs Royal, grown for 6–7 weeks in short or long day conditions, inoculated with three *Phytophthora infestans* isolates

Source	*df*	Analysis of variance mean squares	
		LGR (mm day^{-1})	IP (day)
Growing conditions (GC)	1	202.35***	0.018
Isolate (I)	2	1.95	0.151**
Genotype (G)	5	4.18**	0.124**
GC × I	2	2.95	0.047
GC × G	5	2.70*	0.047
I × G	10	0.67	0.051
GC × I × G	10	0.50	0.011
Error	36	0.94	0.027

Significant at * $P = 0.05$, ** $P = 0.01$, and *** $P = 0.001$, respectively

Discussion

In this study, it was demonstrated that there is a difference in reaction of potato differentials grown in different day length regimes to *P. infestans* isolates. For the first time, it was shown that several isolates avirulent on plants grown in GH and LD conditions appeared to be virulent on plants grown in SD conditions. Determining virulence towards race-specific resistance genes is a prerequisite to understand the response of pathogen populations to resistant cultivars, and therefore to assess the durability of these resistance genes and the performance of resistance management strategies (Andrivon et al. 2011). One of the main activities in potato breeding for resistance to late blight is collecting *P. infestans* isolates representing local populations. These isolates are characterized for phenotypic and genotypic traits, among them for virulence against differential set of potato genotypes having 11 late blight resistance (*R*) genes originated from *Solanum demissum*. The number of methods and different conditions of assessing the virulence of *P. infestans* isolates did not allow comparing the data across laboratories/countries, to have a better understanding on the changes and evolution of this pathogen. One of the goals of the European Concerted Action on Blight EUCABLIGHT was to collate and review existing methods for assessing variation in populations of *P. infestans*, and to test, standardize and publish agreed methods, to create a database accessible via the web of *P. infestans* European isolates. A joint protocol for virulence testing in detached leaf assay was established and a blind ring test involving 12 laboratories and 10 European isolates of the pathogen was conducted. A high level of consensus in the determination of virulence to *R*1, *R*3, *R*4, *R*7, *R*8, *R*10 and *R*11 was achieved among the collaborators, but virulence to *R*2, *R*5 and *R*9 was detected more frequently in some laboratories, essentially from northern Europe and it was concluded that these genes are seems to be highly sensitive to host and environmental conditions (Andrivon et al. 2011). Among many factors that were controlled in the joint protocol day length was not considered. From many years of virulence evaluation, we have noticed that the isolates evaluated in November and December, when the days are shortest during a year in Europe, the pathotypes of some isolates are more complex. For the first time, it was observed in *P. infestans* that a

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Table 5 Resistance to *Phytophthora infestans* expressed as the lesion growth rate (LGR) and incubation period (IP) of potato leaflets of Black's differentials *R*1, *R*3, *R*4, *R*7, *R*11, and cultivar Craigs Royal, grown for 6–7 weeks in short day (SD) or long day (LD) conditions, inoculated with three *Phytophthora infestans* isolates

Growing conditions	Potato genotypes	LGR (mm day^{-1})	Means ranking*	IP (day)	Means ranking
LD	*R*11	4.88	a	2.13	abc
	*R*1	5.36	a	2.36	d
	*R*7	5.75	a	2.20	bcd
	*R*3	5.95	a	2.26	bcd
	*R*4	6.12	a	2.12	ab
	cv. Craigs Royal	7.51	b	2.18	bcd
SD	*R*11	8.55	bc	1.95	a
	*R*7	8.70	bcd	2.26	bcd
	*R*3	9.25	cde	2.35	cd
	cv. Craigs Royal	9.32	cde	2.34	cd
	*R*4	9.85	de	2.24	bcd
	*R*1	10.02	e	2.30	bcd

* Means of LGR or IP followed by the same letter do not differ significantly using Duncan's range test at $P = 0.05$

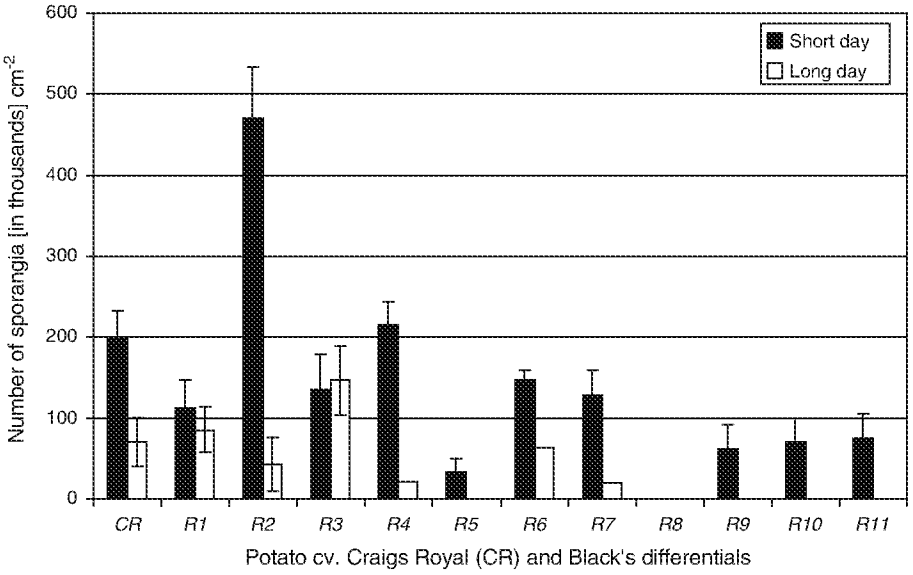


Fig. 2 Sporulation intensity on infected leaflets at 6th day after inoculation with the *Phytophthora infestans* isolate MP585 of potato cv. Craigs Royal and Black's differentials, exposed before inoculation for 7 weeks to short day or long day conditions (the mean ± SD of 3–4 samples)

group of GH avirulent isolates on *R*5, *R*6, *R*8 (seldom but also on *R*9, and *R*10) can behave as virulent on leaflets detached from plants grown in SD conditions, while they remain avirulent or virulent only sporadically for leaflets from LD conditions. Similar effect was reported by Ward and Buzzel (1983) with regard to the light influence on the interaction between soybean genes for resistance and *Phytophthora megasperma* f. sp. *glycinea*. In their studies, several *Rps* genes incompatible with race 4 did not expressed resistance to this race in intact etiolated hypocotyls.

It was revealed that in potato the *R*5, *R*6 and *R*9 differentials posses also the *R*1 gene (Trognitz and Trognitz 2007), and the *R*3 differential contains two closely linked genes, *R*3a and *R*3b (Huang et al. 2004). However, in these studies, *P. infestans* isolates avirulent to the *R*1 and *R*3 were not used; therefore, the obtained results were not biased by these additional genes.

The incompatible interaction of *P. infestans* isolates to Black's differentials *R*1, *R*3, *R*4, *R*7, *R*11 was not tested, because corresponding avirulent isolates were not found in IHAR's collection of the pathogen. Three virulent isolates to all these five *R*-genes were tested, and all these isolates caused symptoms of disease on 20 leaflets of each differential from both short and long day conditions.

The resistance of potato to *P. infestans* depends on many factors. Among them, long day has a strong resistance enhancing effect on potato plants (Trognitz et al. 2009; Colon 1994; Victoria and Thurston 1974). The photoperiod is a significant factor in the expression of quantitative

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resistance to *P. infestans* of some potato genotypes, but not all genotypes of potato react in the same way (Mihovilovich et al. 2010). These findings were confirmed for the resistance to *P. infestans* expressed as the LGR in this study for six different potato genotypes. In studies of Trognitz et al. (2009) blight was significantly ($P < 0.001$) reduced on LD pre-treated, genetically resistant cultivars, whereas the susceptible cultivar Linzer Delikatess developed severe disease symptoms under all conditions (Trognitz et al. 2009).

The ANOVA of LGR values in detached leaf assay showed no significance for isolate, and isolate by day length interaction, in contrast to studies of Mihovilovich et al. (2010), in which the interaction of isolate and day length in the LGR variation played a significant role. The authors performed detached leaflet assay in both SD and LD conditions, in contrast to our test, where the conditions of detached leaflet assay were the same, so only plants, not isolates were exposed to SD and LD conditions. It might be assumed that there is a different effect of day length on the aggressiveness of *P. infestans* isolates (not investigated in our studies). LGR and SI could be enough parameters to assess isolates aggressiveness based on the results of Carlisle et al. (2002) and Flier and Turkensteen (1999). They found that correlations between all combinations of all parameters, area under lesion expansion curve (AULEC)/maximal growth rate (MGR), infection frequency, LP and SI, were significant at $P < 0.05$ up to $P < 0.001$ (with exception of correlation between AULEC/MGR and SI). In our studies, both LGR and SI were significantly favored by SD growing conditions (with exception of SI on differentials *R*1 and *R*3, where the influence of growing conditions was not observed). IE was not influenced by growing conditions and all leaflets were infected by all three isolates used. IP was not influenced by growing conditions, but by isolate and genotype.

The influence of SD and LD conditions was not observed for the infection of the *R*2 Black's differential with all virulent or avirulent *P. infestans* isolates. However, surprisingly many avirulent isolates caused symptoms of infection on leaflets of differentials *R*5, *R*6, and *R*8 grown in SD conditions. Further studies are needed to understand the role of light conditions in qualitative resistance of potato to *P. infestans*. In general, SD growing conditions of potato plants are in favour to stronger infection, expressed as higher LGR, and the stronger SI, but not the IP, after inoculation with virulent *P. infestans* isolates. Potato genotypes differ in sensitivity to different photoperiods. The evaluation of *P. infestans* isolates for their virulence should be conducted on leaflets from plants grown in LD conditions. If this evaluation is being done in late autumn, the day length should be extended with an artificial light. The same should be applied for screening potato parental breeding lines and breeding clones for general resistance to *P. infestans*.

Author contribution Renata Lebecka designed research, conducted research, analyzed data and performed statistical analysis, wrote paper, had primary responsibility for final content. Sylwester Sobkowiak: isolated and provided *Phytophthora infestans* isolates.

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Quantitative Resistance to *Phytophthora infestans* in Potato: A Case Study for QTL Mapping in an Allogamous Plant Species

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ABSTRACT

Phytophthora infestans is the most important fungal pathogen in the cultivated potato (*Solanum tuberosum*). Dominant, race-specific resistance alleles and quantitative resistance–the latter being more important for potato breeding– are found in the germplasm of cultivated and wild potato species. Quantitative trait loci (QTLs) for resistance to two races of *P. infestans* have been mapped in an F_1 progeny of a cross between non-inbred diploid potato parents with multiple alleles. Interval mapping methods based on highly informative restriction fragment length polymorphism markers revealed 11 chromosome segments on 9 potato chromosomes showing significant contrasts between marker genotypic classes. Whereas phenotypically no difference in quantitative resistance response was observed between the two fungal races, QTL mapping identified at least one race specific QT locus. Two QT regions coincided with two small segments on chromosomes *V* and *XII* to which the dominant alleles *R1*, conferring race specific resistance to *P. infestans*, *Rx1* and *Rx2*, both inducing extreme resistance to potato virus X, have been allocated in independent mapping experiments. Some minor QTLs were correlated with genetic loci for specific proteins related to pathogenesis, the expression of which is induced after infection with *P. infestans*.

THE quantitative variation observed for the majority of phenotypic characters in plants is caused by polygenic systems frequently interacting with the environment (JOHANNSEN 1909; NILSSON-EHLE 1909). Genetic loci acting collectively on the expression of a trait have been termed quantitative trait loci, QTLs (GELDERMANN 1975). The quantitative effects of QTLs make them inaccessible to direct Mendelian analysis. However, when marker loci segregating with Mendelian ratios are linked to a QTL, the chromosomal position and the phenotypic contribution of the QTL can be determined (THODAY 1961). The approach is limited by the availability of a sufficient number of Mendelian markers. The advent of molecular markers, such as isoenzymes, and restriction fragment length polymorphism (RFLPs), greatly facilitated the genetic analysis of QTLs, as these markers, particularly RFLPs, are in most cases phenotypically neutral, numerous and environmentally independent. The pioneering QTL mapping experiments in plants making use of molecular markers were performed in maize and tomato on F_2 or backcross populations derived from pure lines (TANKSLEY *et al.* 1982; OSBORN *et al.* 1987; NIENHUIS *et al.* 1987; PATERSON *et al.* 1988; EDWARDS *et al.* 1987, 1992; STUBER *et al.* 1992).

The cultivated potato (*Solanum tuberosum* ssp. *tuberosum*) is a highly heterozygous tetraploid plant showing tetrasomic inheritance. When reduced to the diploid level, the potato is largely self-incompatible. Moreover, the high genetic load present in this vegetatively reproduced species precludes the routine production of inbred lines and hence F_2 or backcrosses among them. Despite these limitations, we show in this study how the detection and mapping of QTLs is possible when segregating F_1 populations from crosses between heterozygous diploid potato lines, a detailed RFLP linkage map (GEBHARDT *et al.* 1991) and highly informative RFLP markers are available.

The trait chosen was quantitative resistance to *Phytophthora infestans*, which is the most important fungal pathogen of the cultivated potato. Epidemics of *P. infestans* cause heavy losses in yield. The fungus is currently controlled by frequent applications of fungicides. However, this creates environmental concern and contributes to the emergence of resistant isolates (COHEN and REUVENI 1983). Since the beginning of this century, much effort has been devoted to breeding for resistance to *P. infestans* (reviewed in WASTIE 1991). A number of dominant alleles conferring race-specific resistance controlled by single loci (*R* genes) have been introduced into cultivars from the wild species *Solanum demissum*. This type of resistance was overcome soon by new races of the fungus having virulence alleles compatible with the resistance alleles of the cultivars. Current breeding strategies concentrate, therefore, on improving horizontal, race-unspecific resistance which is polygenically inherited and for which selection is difficult (WASTIE

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1991). The relationships between the genetic factors controlling qualitative and quantitative resistance to *P. infestans* are unknown.

The identification of the map positions and of RFLP marker probes linked to qualitative (LEONARDS-SCHIPPERS *et al.* 1992) and quantitative resistance genes is, therefore, an essential step toward identifying better breeding strategies for *P. infestans* resistance based on marker-assisted selection.

MATERIALS AND METHODS

Plant material: Crossing the diploid potato lines H82.368/3 (line 49 in GEBHARDT *et al.* 1989) as female parent and H80.696/4 (line 40, GEBHARDT *et al.* 1989 and BARONE *et al.* 1990) as male parent resulted in 197 F_1 plants. H82.368/3 is referred to as P49 in this report and was highly resistant to both *P. infestans* races 0 and 1 (mean percent infection 3.5 ± 3%) under the conditions of the infection test. H80.696/4 is referred to as P40 and was susceptible to races 0 and 1 (mean percent infection 65 ± 13%). Parents and progeny used for quantitative resistance tests to *P. infestans* were grown in a controlled environment chamber (BBC) at 17°, 14 hr light and between *ca.* 70 and 100% relative humidity.

For DNA extraction, *ca.* 10 g leaves and shoots were harvested from plants grown in the greenhouse, frozen in liquid nitrogen, freeze dried and stored at −20°.

***P. infestans* races:** *P. infestans* race 0 was used for the test series T0a and T0b. Race 0 was obtained originally from H. R. HOHL (University of Zürich, Switzerland). *P. infestans* race 1 was used in the test series T1a and T1b. It was provided by B. SCHÖBER (Biologische Bundesanstalt, Braunschweig, Germany). Both races were verified on the Netherlands differential test set kindly provided by L. J. TURKENSTEEN (DLO Research Institute for Plant Protection, Wageningen, The Netherlands). *P. infestans* races were propagated as described in LEONARDS-SCHIPPERS *et al.* (1992).

RFLP analysis: DNA extraction, restriction digests, electrophoresis, blotting and hybridization procedures were as described previously (GEBHARDT *et al.* 1989; LEONARDS-SCHIPPERS *et al.* 1992). The restriction enzymes *Taq*I, *Rsa*I and *Alu*I (Boehringer Mannheim; GIBCO/BRL, Gaithersburg, Maryland) were used according to the supplier's instructions. Filters onto which restricted genomic DNA of parents and F_1 plants had been transferred were hybridized to the radiolabeled inserts of the plasmids of 102 RFLP marker clones mapping to the 12 linkage groups of potato (GEBHARDT *et al.* 1991; unpublished results from this laboratory). CP and GP marker probes were derived from potato cDNA and genomic DNA, respectively. TG markers originated from genomic tomato DNA and were kindly provided by S. D. TANKSLEY (Cornell University, Ithaca, New York). *β-Gluc* was mapped with a cDNA clone coding for 1,3-β-glucanase of potato and was provided by E. KOMBRINK (MPI für Züchtungsforschung, Köln, Germany). *PC116* and *prp1* were mapped with two cDNA clones of potato which had been identified by differentially screening (infected *vs.* noninfected) a cDNA library of potato leaves infected with an incompatible race of *P. infestans* (TAYLOR *et al.* 1990). The *WUN1* locus is based on a cDNA clone of potato whose mRNA is induced after wounding (LOGEMANN *et al.* 1988).

Test for quantitative resistance to *P. infestans*: A leaf disc bioassay was used (GIEFFERS *et al.* 1989; LEONARDS-SCHIPPERS *et al.* 1992) which was a modification of the leaf disc bioassay developed by HODGSON (1961). In each of four test series eight leaf discs per genotype were inoculated, each with three 10-µl aliquots of a suspension adjusted to 3×10^4 sporangia and 4×10^4 zoospores per ml. The eight leaf discs were cut from the leaflets of the same or a directly adjacent fully expanded leaf at the third internode from the top of the plant. The same plants were used for all four test series (T0a, T0b, T1a, T1b). The approximate age of the plants was 8 weeks for test series T0b, 14 weeks for T0a, 15 weeks for T1b and 17 weeks for T1a. After 3–4 days of incubation at 17°, 14-hr light period, the leaf discs were scored for percentage of infected leaf disc areas using the scale shown in Figure 1.

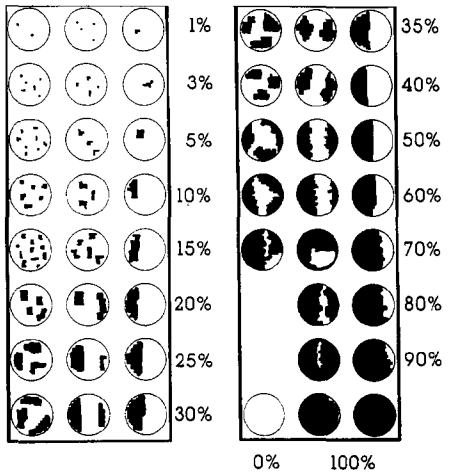


FIGURE 1.—Evaluation chart for measuring percent infection to *P. infestans* in the leaf disc assay. Infected areas on the leaf discs are shown in black.

Data analysis: The segregation of RFLP alleles was analyzed as presence *vs.* absence of specific restriction fragments in the parents and the F_1 progeny. Linkage among all RFLP alleles scored and the order of linked RFLP loci was determined based on the methods described by RITTER *et al.* (1990) and on software developed by E. RITTER. For each chromosome two linkage groups were constructed based on heterozygous RFLP loci with alleles specific for the female (P49) or for the male (P40) parent. These alleles are expected to segregate with a 1:1 ratio in the F_1 offspring. The alternative alleles at each heterozygous RFLP locus in P49 and P40, respectively, were scored as 1 and 0. If both parents possessed different alleles at an RFLP locus, four genotypic classes in the F_1 could then be identified as 11, 10, 01 and 00, equivalent to the four allelic states A_1A_3, A_1A_4, A_2A_3 and A_2A_4as shown in Figure 2. Alleles at heterozygous RFLP loci which are identical in both parents are expected to segregate with a 3:1 ratio (presence *vs.* absence) in the F_1 offspring. These alleles were omitted from the QTL analysis.

A generalized genetic model applicable to the segregation of a quantitative trait locus (QTL) in an F_1 offspring derived from crossing non-inbred parents is shown in Figure 2. The four QT alleles, Q_1, Q_2 (present in P1) and Q_3, Q_4 (present in P2) constitute the four genotypic QT classes Q_1Q_3, Q_1Q_4, Q_2Q_3, Q_2Q_4 in the F_1 generation which are expected to segregate with a 1:1:1:1 ratio. The QTL is flanked by two RFLP marker loci *A* and *B*, each also with four RFLP alleles A_1, A_2, A_3, A_4 and B_1, B_2, B_3, B_4, respectively. From the 16 genotypic classes formed by the RFLP alleles of *A* and *B* in the F_1 generation (4 non-recombinant and 12 recombinant classes) only the non-recombinant classes I, II, III and IV are shown in Figure 2.

QTLs were mapped using least squares interval mapping methods developed for backcross progeny derived from inbred lines (KNAPP *et al.* 1990; KNAPP and BRIDGES 1990). The two separate genetic maps (see above), one built with alleles from

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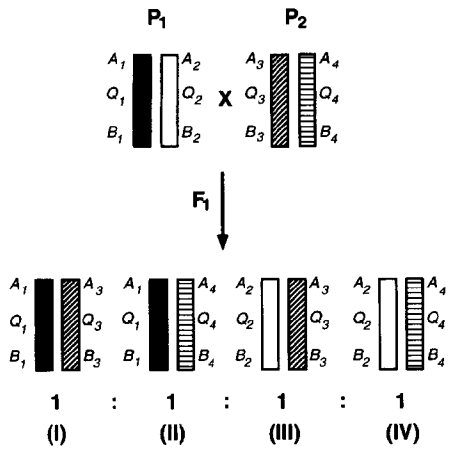


FIGURE 2.—Genetic model for a QTL (Q) flanked by the marker loci A and B in the heterozygous parents P1 and P2 and in the F_1 generation derived from them.

P1 (P49) and the other one with alleles from P2 (P40), are equivalent to reciprocal backcross maps. QTLs were interval-mapped separately in each of the two genetic maps. The hypothesis of no QTL was tested against the hypothesis of one QTL within each marker interval based on differences between phenotypic means of marker genotypic classes.

The two non-recombinant marker genotypic classes of the backcross model ($A_1A_2B_1B_2$ and $A_1A_1B_1B_1$) were substituted by the pooled marker genotypic classes I plus II and III plus IV (Figure 2) for marker intervals derived from P1 (P49). The two non-recombinant marker genotypic classes for marker intervals derived from P2 (P40) were formed by the pooled marker genotypic classes I plus III and II plus IV (Figure 2). The two recombinant marker genotypic classes of the backcross model ($A_1A_1B_2B_1$ and $A_2A_1B_1B_1$) were substituted accordingly by the marker genotypic classes recombinant among the alleles A_1, A_2, B_1 and B_2 of the P1 parent and the marker genotypic classes recombinant among the alleles A_3, A_4, B_3 and B_4 of the P2 parent, respectively. The QTL effects we estimated in each marker interval resulted, therefore, from the difference between the QT alleles Q_1 and Q_2 of parent P1 in the presence of either Q_3 or Q_4 and, vice versa, from the difference between the QT alleles Q_3 and Q_4 of parent P2 in the presence of either Q_1 or Q_2.

Parameters were estimated for the full model assuming one QTL between a pair of markers and the reduced model assuming no QTL between the same pair of markers using least square methods in the NLIN procedure of the SAS/STAT software (KNAPP and BRIDGES 1990; SAS Institute Inc. 1989). The likelihood ratio L for testing the hypothesis of no QTL is

$$L = \frac{(\mathrm{SSE}_R - \mathrm{SSE}_F)/(\mathrm{d.f.}_R - \mathrm{d.f.}_F)}{\mathrm{SSE}_F/\mathrm{d.f.}_F}$$

where SSE_F and SSE_R are the error sum of squares derived from the NLIN procedure for the full model and the reduced model, respectively, and $\mathrm{d.f.}_F$ and $\mathrm{d.f.}_R$ are the degrees of freedom for the full model and the reduced model, respectively. $\mathrm{d.f.}_F$ and $\mathrm{d.f.}_R$ are given by $N - P_F$ and $N - P_R$, where N is the number of valid observations and P_F and P_R are the numbers of the estimated parameters for the full model and the reduced model, respectively.

L is distributed as an F random variable, so that if $L > F_{\alpha,\mathrm{d.f.}N,\mathrm{d.f.}F,0}$, where $F_{\alpha,\mathrm{d.f.}N,\mathrm{d.f.}F,0}$ is a critical value from the central F-distribution and $\mathrm{d.f.}_N$ is $\mathrm{d.f.}_R - \mathrm{d.f.}_F$, the hypothesis of no QTL can be rejected. The P value for a likelihood ratio obtained is given by 1 - PROBF (L,$\mathrm{d.f.}_N$,$\mathrm{d.f.}_F$,0), where PROBF is an SAS function returning the probability that an observation of an F distribution is less than or equal to L (SAS Institute Inc. 1990). The hypothesis of no QTL was rejected for $P \leq 0.05$ ($\alpha \leq 5\%$).

The statistical software SPSS for Windows, version 5.0 (SPSS Inc. 1992) was used for the analysis of normal distributions and transformation of the quantitative resistance data. Analysis of variance was performed with the procedure ONEWAY of the same software. Paired t-tests were performed using SAS programs (SAS Institute Inc. 1991).

RESULTS

Quantitative resistance to races 0 and 1 of *P. infestans* was determined in 197 F_1 plants. Four test series were performed on the parents and the F_1 progeny, two (T0a and T0b) with race 0 and two (T1a and T1b) with race 1. Three additional datasets (T0, T1 and T01) were created by averaging the mean resistance values obtained for each genotype in test series T0a and T0b (T0), in T1a and T1b (T1) and in all four test series (T01). After the transformation

$$x' = \arctan\left((x/110)/\sqrt{1 - ((x/110) \times (x/110))}\right)$$
$$= \arcsin(x/110) \qquad \text{with} \quad x = \%\ \text{infection}$$

all datasets except the dataset T0b were normally distributed according to the criteria of the Kolmogoroff-Smirnoff test (LILLIEFORS 1967; SACHS 1992). All seven distributions of transformed values exhibited negative skewness and also negative kurtosis (SACHS 1992). The statistical parameters of the seven datasets are shown in Table 1 and the distributions of the combined datasets T0, T1, and T01 are shown in Figure 3. The resistance levels of the parents P49 and P40 are also indicated in Table 1 and Figure 3. Whereas the resistant parent P49 was positioned at the left hand end of the distributions, the mean resistance of the susceptible parent P40 was close to the mean of the population. Based on the phenotype of quantitative resistance to *P. infestans*, no difference between race 1 and race 0 was observed.

The segregation of RFLP alleles was analyzed in 189 of the 197 F_1 individuals (8 were not available due to practical reasons) of the same population and in the parents P49 and P40. Southern blots were probed with a set of RFLP markers selected from the potato RFLP map (GEBHARDT *et al.* 1991; unpublished results from this laboratory). The markers were chosen according to equidistant map positions and informativity for both parents, taking into consideration the coverage of both parental genomes with RFLP marker loci. Two linkage maps were constructed: one with 77 RFLP alleles descending from P40 covering 1025 cM and another with 68 RFLP alleles descending from P49 and covering 1028 cM (Figure 4). The mean interval length was 14 cM. Thirty-four of 111 mapped loci (31%) showed heterozygous RFLP alleles differing in both parents and allowed, therefore, the identification of the four possible geno-

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TABLE 1

Statistical parameters of transformed phenotypic values of the seven datasets in the parents P40, P49 and in the F_1 progeny

Parameter	T0a	T0b	T0	T1a	T1b	T1	T01
Mean P40	0.720	0.478	0.594	0.591	0.555	0.581	0.588
Mean P49	0.077	0.035	0.056	0.044	0.039	0.042	0.049
Mean F_1	0.642	0.638	0.639	0.645	0.659	0.652	0.646
Median	0.650	0.620	0.633	0.655	0.700	0.693	0.654
Standard deviation	0.271	0.286	0.224	0.305	0.275	0.249	0.201
Skewness	−0.258	−0.241	−0.151	−0.135	−0.234	−0.283	−0.182
Kurtosis	−0.652	−0.815	−0.533	−0.944	−0.756	−0.655	−0.597
Kolmogoroff-Smirnoff level of significance	>0.2	0.02	>0.2	>0.2	>0.2	>0.2	>0.2

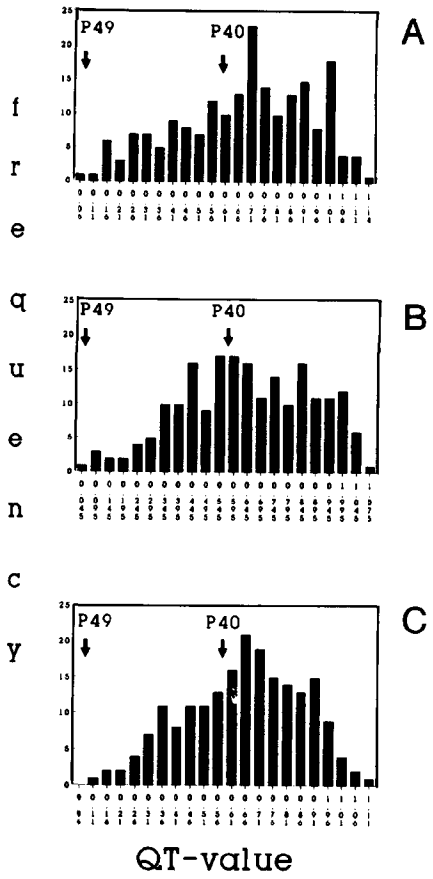


FIGURE 3.—Frequency distribution of quantitative resistance to *P. infestans* in the F_1 progeny derived from the cross P49 × P40 in the combined data sets T1 (A), T0 (B) and T01 (C). Transformed (x' = arcsin (x/110)) phenotypic values are shown on the x-axis. The positions of the mean resistance values of the parents are indicated by arrows.

typic classes A_1A_3, A_1A_4, A_2A_3 and A_2A_4 in the F_1 population (see Figure 2). Each chromosome was covered by 2–10 marker intervals with the exception of chromosome *XII* of parent P49 for which no informative marker could be found. All 13 markers of chromosome *XII* tested appeared to have homozygous RFLP alleles for P49 whereas 11 heterozygous RFLP alleles from the same markers segregated for P40 (Figure 4).

Incompatible *S* alleles at the *S* locus on chromosome *I* (GEBHARDT *et al.* 1991) were also active in the cross. This was evident from the observation of strong deviations from the expected 1:1 segregation ratios of the RFLP alleles descending from the male parent P40 on chromosome *I* (data not shown).

The 1015 marker intervals (145 intervals for the two maps, multiplied by the 7 datasets) were tested for the presence of a QTL effect. Seventy intervals of those were significant at $P \leq 0.05$. A QTL effect was detectable in most cases in at least two intervals adjacent to each other and several marker intervals appeared significant in more than one dataset. Taking this redundancy into account, eight segments were identified on chromosomes *II, III, IV, V, VII, IX, XI* and *XII* of the susceptible parent P40 and six segments on chromosomes *III, IV, V, VI* and *VII* of the highly resistant parent P49 (Figure 4), which showed QTL effects at various levels of significance.

With the exception of chromosomes *IV* and *V*, on which both parents showed effects in similar chromosomal segments, all QTL effects were detected in only one parent, preferentially in the susceptible parent P40 (Figure 4).

The chromosomal regions harboring QTLs were further characterized by defining 19 putative QT loci for *P. infestans* resistance (*Pi* loci, Figure 4, Table 2): *Pi* loci were allocated to the positions of maxima of the likelihood ratio curves within marker-brackets. In segments with significant consecutive marker intervals a *Pi* locus was assigned to the interval with the highest level of significance with the exception of two segments on chromosomes *IV* and *V* of the P40 map comprising three and two intervals, respectively, of a very high ($P < 0.001$) level of significance. (*GP180(a)-TG123-GP261(a)-TG62* on chromosome *IV*, *GP186-GP21-GP179* on chromosome *V*). As the *P* values were very similar for these intervals, three and two, respectively, *Pi* loci were assigned to these regions (*Pi1(a)*, *Pi1(b)*, *Pi1(c)* on chromosome *IV*, *Pi01(a)*, *Pi01(b)* on chromosome *V*). These *Pi* "loci" in adjacent intervals can be due to a single locus having a strong QTL effect across several marker loci or due to an unknown number of linked QTLs. The *Pi* loci and the phenotypic contrasts A as well as the *P* values associated with them in the seven datasets are listed in Table 2. Three types of *Pi* loci were discriminated based on their significance across test series and fungal races. The

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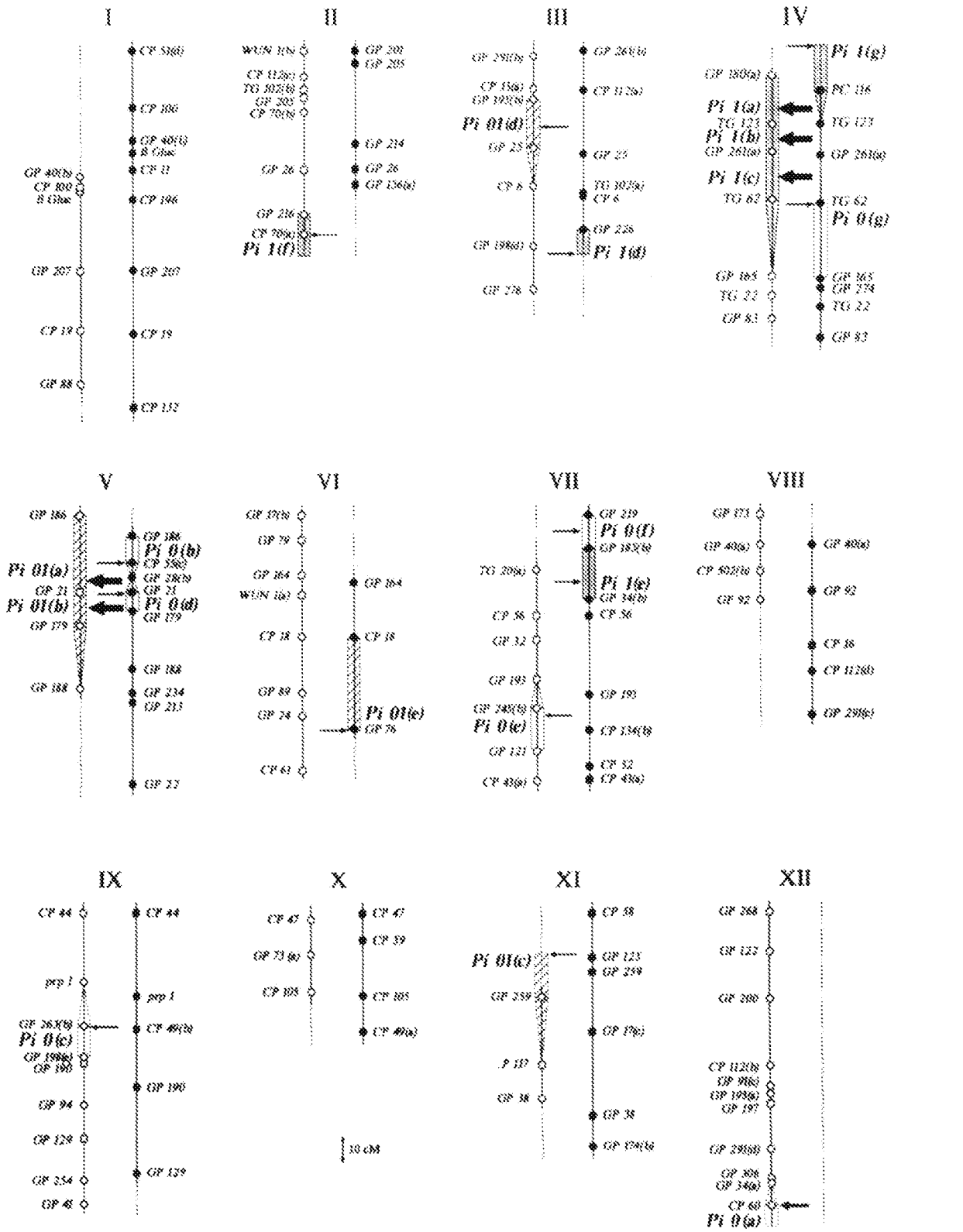


FIGURE 4.—The map positions of *P. infestans* QT loci on the RFLP linkage groups derived from the parent P40 (open circles) and P49 (solid circles). Linkage group XII of P49 is missing as no informative markers were found (see text). Marker intervals having significant phenotypic effects are shown as rectangular areas, slashed for *Pi01* loci, shaded for *Pi1* loci and white for *Pi0* loci. Neighboring intervals with decreased, but still significant *P* values are shown as triangular areas. Arrows (thin, $0.05 < P < 0.01$; medium, $0.01 > P > 0.001$; thick, $P < 0.001$) indicate the positions of *Pi* loci within marker intervals based on the *P* values underlined in Table 2.

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first type are the five race-unspecific *Pi01* loci (*Pi01(a)-Pi01(e)*): they are characterized as inducing significant phenotypic contrasts at $P \leq 0.05$ in at least one test series with race 1 and race 0. The *P* values in the T01 dataset, combining all four test series, were below threshold irrespective of whether or not all individual test series were significant. The strongest effect ($P < 0.001$) was found for *Pi01(a)* and *Pi01(b)* in two consecutive intervals on chromosome *V* of parent P40.

The second type is represented by the seven *Pi1* loci (*Pi1(a)-Pi1(g)*). Effects are significant at $P \leq 0.05$ in the two test series T1a and/or T1b performed with race 1 or in the dataset T1 combined from them. Specificity for race 1 was particularly evident for the QTL effects with the highest significance in the whole dataset in three adjacent marker intervals on chromosome *IV* of parent P40 (*Pi1(a)*, *Pi1(b)* and *Pi1(c)*). Phenotypic contrasts caused by QT alleles of P49 in the same genomic region (*Pi1(g)* and *Pi0(g)*) were also detectable, although at a low level of significance.

The third type are the seven QT loci named *Pi0(a)–Pi0(g)*. The criterion for a *Pi0* QT locus was a *P* value below 0.05 in the test series T0a and/or T0b performed with race 0 or in the combined dataset T0. The most significant *Pi0*-QTL (*Pi0(a)*, $P = 0.01$) showing specificity to race 0 was found on chromosome *XII* of P40 close to the marker *CP60*.

In 8 of 19 cases, a phenotypic effect was detectable in only one individual test series and not in any of the combined datasets (*Pi1(e)*, *Pi1(f)*, *Pi1(g)*, *Pi0(b)*, *Pi0(c)*, *Pi0(d)*, *Pi0(f)*, *Pi0(g)*)). With the exception of *Pi1(e)* which was significant at $P = 0.01$, levels of significance were low ($0.01 < P < 0.05$). Considering the large number of tests performed, some of those phenotypic effects may have arisen by chance alone (false positives). Alternatively, they could also be the result of QTLs having small effects combined with race specificity or of particular environmental or developmental conditions affecting resistance only during a singular test series.

The position of four QT loci (*Pi1(d)* on chromosome *III*, *Pi1(g)* on chromosome *IV*, *Pi01(c)* on chromosome *XI*, *Pi0(a)* on chromosome *XII*) was based on the highest level of significance found in the "interval" ($r = 0.5$) distal to the last marker of the linkage group. In three of the four cases also the adjacent internal interval showed significant phenotypic contrasts with the same or decreasing effect (Figure 4).

The traditional approach to QTL analysis is based on the detection of phenotypic contrasts between genotypic classes at single marker loci, whereas the analysis presented in Figure 4 and Table 2 is based on one of the more recently developed flanking marker models (KNAPP *et al.* 1990; LANDER and BOTSTEIN 1989). In the traditional approach, the recombination frequency between a marker locus and a QTL is an unknown variable as compared to interval mapping of QTLs according to KNAPP *et al.* (1990), where the recombination frequencies r_1 and r_2 of the QTL to the flanking markers are estimated.

To compare the two approaches, paired *t*-tests were performed to detect significant phenotypic contrasts between the two alternative RFLP alleles at each RFLP locus individually scored in the parents P40 and P49, respectively. At the 5% level of significance, essentially the marker loci in those chromosomal segments showed QTL effects which had been identified by using the interval mapping approach. Few additional marker loci were significant at $0.01 < P < 0.05$ (data not shown).

The phenotypic contrasts based on which QTLs were mapped to potato chromosomes are the results of the action of up to four different alleles at any QT locus (Figure 2). The effects of individual QT alleles, dominance or additivity, are not revealed by the method used for identifying the map positions. However, when the four marker genotype classes of an RFLP locus could be identified in the F_1 offspring, it was possible to examine phenotypic effects of allelic combinations at a linked QT locus.

Analysis of variance was performed between the phenotypic means (transformed values) of the marker genotype classes A_1A_3, A_1A_4, A_2A_3 and A_2A_4 at informative marker loci linked to the QTLs on chromosomes *III*, *IV*, *V*, *IX* and *XI* (Table 3). Significant differences ($P \leq 0.05$) among classes were found for all marker loci except for *GP259*. When compared among each other and with the population mean, favorable as well as unfavorable allelic combinations were identified. For example, the allelic combination A_2A_4 at the RFLP locus *GP25* linked to *Pi01(d)* on chromosome *III* increased resistance (decreased % infection), whereas A_1A_3 decreased it (increased % infection) with A_1A_4 and A_2A_3 having no effect. The situation is best explained by additive effects without interaction in the F_1 of two positive alleles A_2 (P49) and A_4 (P40) and two negative alleles A_1 (P49) and A_3 (P40). The difference between the QT alleles of P49, evident in this analysis, appeared not to be significant with the interval mapping procedure described above. By comparing the phenotypic means at the linked marker loci on chromosome *IV*, a similar qualitative relationship could be deduced for the whole chromosome segment harboring the highly significant QT loci *Pi1(a)*, *Pi1(b)* and *Pi1(c)*.

In the QTL region on chromosome *V*, positive and negative QT alleles of P40 that are linked to the marker alleles A_4 and A_3, respectively, appeared to be dominant to the QT alleles of P49. At the QT locus *Pi0(c)* on chromosome *IX*, the allele combination A_1A_4 at the marker locus *prp1* decreased resistance compared to the population mean whereas the other three genotypes had no significant effect. Negative, heterozygous recessive QT alleles present in both parents may have caused the observed phenotype.

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TABLE 2

Contrasts *A* (based on transformed phenotypic values of percent infection) and *P* values of *P. infestans* QT loci

QTL	Chromosome no./parent	Test series and datasets: T0a *A*	T0a *P*	T0b *A*	T0b *P*	T0 *A*	T0 *P*	T1a *A*	T1a *P*	T1b *A*	T1b *P*	T1 *A*	T1 *P*	T01 *A*	T01 *P*
Pi01(a)	*V*/P40	0.230[b]	0.0031	0.190	0.0296	0.207	0.0008	0.136	0.0575	0.194	0.0102	0.156	0.0051	0.178	0.003[a]
Pi01(b)	*V*/P40	0.150	0.0068	0.060	0.4873	0.109	0.0205	0.133	0.0442	0.162	0.0033	0.153	0.0022	0.135	0.0006
Pi01(c)	*XI*/P40	0.157	0.0232	0.085	0.2304	0.126	0.0680	0.176	0.0248	0.110	0.0811	0.159	0.0114	0.142	0.0157
Pi01(d)	*III*/P40	0.148	0.0069	0.001	0.9996	0.078	0.1733	0.213	0.0014	0.077	0.3085	0.138	0.0138	0.114	0.0167
Pi01(e)	*VI*/P49	0.117	0.0384	0.121	0.5759	0.075	0.1484	0.154	0.0115	0.065	0.3680	0.104	0.0452	0.095	0.0227
Pi1(a)	*IV*/P40	0.055	0.4557	0.072	0.3142	0.068	0.1698	0.231	0.0001	0.133	0.0147	0.179	0.0002	0.119	0.0018
Pi1(b)	*IV*/P40	0.073	0.2459	0.071	0.3255	0.069	0.1948	0.233	0.0000	0.140	0.0142	0.184	0.0001	0.129	0.0012
Pi1(c)	*IV*/P40	0.078	0.2255	0.023	0.8978	0.051	0.3933	0.252	0.0000	0.138	0.0265	0.193	0.0001	0.122	0.0042
Pi1(d)	*III*/P49	0.108	0.2120	0.112	0.2412	0.017	0.9038	0.196	0.0066	0.125	0.1044	0.156	0.0117	0.084	0.2105
Pi1(e)	*VII*/P49	0.104	0.0691	0.019	0.9214	0.053	0.4290	0.029	0.8369	0.147	0.0102	0.083	0.1870	0.060	0.2723
Pi1(f)	*II*/P40	0.054	0.6078	0.011	0.9788	0.026	0.8393	0.159	0.0202	0.038	0.7705	0.097	0.1231	0.053	0.3874
Pi1(g)	*IV*/P49	0.113	0.1487	0.032	0.8542	0.081	0.2196	0.176	0.0342	0.054	0.5165	0.124	0.0710	0.110	0.0512
Pi0(a)	*XII*/P40	0.097	0.1149	0.130	0.0179	0.107	0.0091	0.108	0.1048	0.066	0.3209	0.108	0.1076	0.091	0.0190
Pi0(b)	*V*/P49	0.044	0.7146	0.160	0.0124	0.097	0.0902	0.068	0.4778	0.041	0.7539	0.028	0.8479	0.030	0.7547
Pi0(c)	*IX*/P40	0.012	0.9581	0.118	0.0272	0.064	0.1640	0.038	0.7280	0.028	0.8050	0.021	0.8602	0.041	0.4232
Pi0(d)	*V*/P49	0.034	0.8607	0.120	0.0330	0.065	0.1916	0.050	0.5923	0.020	0.9021	0.036	0.6693	0.019	0.8428
Pi0(e)	*VII*/P40	0.064	0.3816	0.119	0.0385	0.090	0.0446	0.065	0.4009	0.039	0.7055	0.055	0.4212	0.064	0.1895
Pi0(f)	*VII*/P49	0.125	0.0369	0.047	0.6456	0.065	0.2303	0.040	0.7456	0.111	0.0734	0.072	0.2428	0.064	0.1777
Pi0(g)	*IV*/P49	0.108	0.0492	0.036	0.7523	0.041	0.5516	0.096	0.1510	0.042	0.6802	0.075	0.1908	0.055	0.2580

[a] Numbers underlined indicate those *P* values based on which the map positions of the QTLs in Figure 4 were allocated.
[b] An *A* value of 0.1 is approximately equivalent to a 10% difference between means of percent infected leaf disc area. Infection ranges are given in Figure 3.

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TABLE 3

Analysis of variance among phenotypic means (transformed values) of the four marker genotype classes at RFLP loci linked to *P. infestans* QTLs

Locus	Chromosome	Dataset	F_1 marker genotype classes: P49 P40 A_1A_3	P49 P40 A_1A_4	P49 P40 A_2A_3	P49 P40 A_2A_4	Population mean	P
GP25	*III*	T01	0.714(49)[a]	0.631(36)	0.652(38)	0.414 (9)	0.641(180)	0.0006
			−	+/−	+/−	+		
GP180(a)/PC116[b]	*IV*	T1	0.716 (64)	0.643(26)	0.668(47)	0.520(29)	0.657(182)	0.0122
TG123	*IV*	T1	0.736 (64)	0.568(25)	0.684(47)	0.531(28)	0.657(182)	0.0011
GP261(a)	*IV*	T1	0.730 (65)	0.573(26)	0.671(42)	0.528(30)	0.657(182)	0.0013
TG62	*IV*	T1	0.732 (56)	0.601(28)	0.683(41)	0.509(29)	0.657(182)	0.0011
			−	+/−	−/+	+		
GP186	*V*	T0	0.680 (35)	0.558(26)	0.674(43)	0.416 (4)	0.639(188)	0.0514
GP21	*V*	T01	0.692 (37)	0.570(39)	0.719(37)	0.598(42)	0.631(180)	0.0050
GP179	*V*	T01	0.654 (42)	0.602(28)	0.725(37)	0.565(47)	0.641(180)	0.0035
			−	+	−	+		
prp1	*IX*	T0	0.607 (48)	0.734(40)	0.597(28)	0.638(53)	0.639(188)	0.0402
			+/−	−	+/−	+/−		
GP259	*XI*	T01	0.654 (44)	0.560(18)	0.669(70)	0.582(26)	0.641(180)	0.1401

[a] Numbers in parenthesis are numbers of observations. Missing values cause the differences between no. of observations for the population mean and the sum of observations for the genotype classes.

[b] The alleles A_1 and A_2 of P49 are from *GP180(a)* and alleles A_3 and A_4 of P40 are from *PC116* in this case (see Figure 4).

With the exception of the loci *GP21* and *GP179*, the frequencies of the marker genotype classes all deviated significantly ($P < 0.05$) from the expected 1:1:1:1 segregation ratio. The genotypic classes that increased susceptibility were always in excess to those that increased resistance (Table 3). This was particularly evident for the positive allele combinations A_2A_4 at the loci *GP25* (chromosome *III*) and *GP186* (chromosome *V*) for which only 9 and 4 genotypes, respectively, were identified in the F_1 progeny as compared to the approximately 33 and 27 expected.

DISCUSSION

Mapping QTLs in F_1 progenies of non-inbred parents: When a backcross derived from homozygous inbred parents is used in QTL analysis, phenotypic contrasts are formed between one homozygous and one heterozygous marker genotype class. In QTL experiments based on doubled haploid and homozygous inbred lines, respectively, the two classes being compared carry different alleles in the homozygous state. In the F_1 progeny used in this study, contrasts were formed between two heterozygous marker genotype classes, each carrying one of the two alternative alleles of one parent and either one or the other allele of the second parent. The phenotypic contrasts observed resulted, therefore, from the difference between the two alternative QT alleles of the first parent in the presence of the effects of the QT alleles in the second parent. Unlike in progenies derived from pure lines, the detection of QTLs in heterozygous F_1 progenies depends on the within parent variation of QT alleles.

We have chosen the 5% error probability level for accepting the hypothesis of a QTL being present in a marker interval. Although in total more than 1000 intervals have been tested, a large proportion of those was not independent as they were genetically linked. When intervals separated by 50 cM are considered independent and when the two parental maps are treated separately, we estimate that about 40 intervals within the 2000 cM scanned (*ca.* 1000 cM for each parent) were independent. When the four experiments performed (T0a, T0b, T1a and T1b) were also considered independent (datasets T0, T1 and T01 were not independent), approximately 160 (4×40) independent interval tests have been performed resulting in the identification of at least 13 QTL regions (19 *Pi* loci) on 13 different chromosomes (Figure 4). At the 5% level of significance, 8 of 160 tests may appear significant by chance alone (false positives, type I error). We point out, however, that the two QTL regions of the parent P40 on chromosomes *IV* and *V* were significant at the 0.1% level. Two QTL regions of parent P49 that were significant only at the 5% level, were supported by the fact that they were located in the same segment of chromosomes *IV* and *V* as the highly significant QTLs of P40. Three QTL regions could be detected at the 1% (*Pi01(d)*) and 5% (*Pi01(c)*, *Pi01(e)*) level, respectively, in two experiments involving two different fungal races. Two further QTL regions (*Pi1(d)*, *Pi0(a)*) that have been detected with only one race of the fungus, were significant at the 1% level. The remaining four QTL regions (*Pi1(f)*, *Pi0(c)*, *Pi0(e)*, *Pi0(f)*) were of low significance and detectable in one experiment only. There is a high probability that at least one of those QTLs is a false positive.

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The aim of our experiment was the identification of regions in the potato genome possibly involved in quantitative resistance to *P. infestans* that can be tested for reproducibility and size of effects in different genetic backgrounds. It was, therefore, more important to avoid a type II error (discarding an existing difference based on its low significance) than a type I error (declaring a QTL which is a false positive). For this reason we have considered all chromosome segments significant at the 5% level or below that are reported in this paper as possible targets for marker assisted selection experiments. The 5% level of significance was used by EDWARDS *et al.* (1992) for detecting QTLs in maize, whereas the more stringent 0.1% level was chosen by PATERSON *et al.* (1988) and STUBER *et al.* (1992) for mapping QTLs in an interspecific cross of tomato and in highly heterotic progeny of maize inbreds, respectively.

The largest phenotypic contrasts in our F_1 progeny were found for the QT alleles of the susceptible parent P40 in the highly significant QTL regions ($P < 0.001$) on chromosomes *IV* and *V*. Moreover, more QTL effects were found comparing alleles of P40 than comparing alleles of P49. Parent P40 was in interspecific hybrid between *S. tuberosum* and the wild species *Solanum spegazzinii* with a high degree of heterozygosity (GEBHARDT *et al.* 1989). The hybrid nature of P40 may, in part, explain its performance in this QTL analysis.

As highly informative marker loci were available that allowed the discrimination of four genotypic classes in the F_1 offspring, the relative quality of linked QT alleles and their allelic combinations could be evaluated. Combinations of marker alleles have been detected in our experiment that increased or decreased resistance. This type of information can be used for marker assisted selection. Some of the more resistant genotypes were underrepresented in the F_1 population indicating a selective disadvantage. This observation may explain–besides overdominance of susceptibility alleles–the shift of the phenotypic distribution in the F_1 toward susceptibility compared to the mid parent mean, which is a general observation in potato progenies segregating for field resistance to *P. infestans* [ROSS (1986) and references therein].

QTL mapping is often done by estimating differences between means of individual marker genotypes [for example, EDWARDS *et al.* (1987)]. This underestimates the effect of a QTL unless the QTL is very close to the marker locus. Interval mapping of QTLs provides information on the genetic distance of the QTL from flanking markers. The recombination values between pairs of marker loci and a QTL have, however, an unknown variance (KNAPP *et al.* 1990). In our case applying to the same set of data the "traditional" approach, that is comparing mean values of marker genotype classes at single marker loci, led to the identification of the same QTL regions for *P. infestans* resistance. STUBER *et al.* (1992) obtained similar results using both methods of analysis in mapping QTLs in maize.

Race specificity of QTLs for *P. infestans*: Quantitative resistance to *P. infestans* is described as race-unspecific (VANDERPLANK 1984). In accordance with this, the phenotypic distribution of resistance in the F_1 was similar for the two fungal races tested (Figure 3). Dissecting the distributions into QTLs revealed, however, differences between the races. In particular, the most significant QTL region (*Pi1(a)*, *Pi1(b)*, *Pi1(c)*) involving three marker intervals of P40 on chromosome *IV*, was detected only with race 1. Neither of the parents P40 and P49 carried any *R* gene conferring race specific resistance, as both were susceptible, to different degrees, to race 0. Moreover, the dominant allele *R1*, inducing resistance to all fungal races not homozygous for the virulence allele *v1*, has been mapped to potato chromosome *V* (LEONARDS-SCHIPPERS *et al.* 1992). The race specificity associated with the QTL region on chromosome *IV* is, therefore, controlled by other genetic factors than the race specificity of the *R1* allele.

Race specificity may also be involved in the *P. infestans* QT loci *Pi1(d)–Pi1(g)* and *Pi0(a)–Pi0(g)*. Their effect appeared significant, however, only in one of four test series. Five of the seven *Pi0* loci (*Pi0(a)–Pi0(e)*) were, for example, significant in the test T0b. The plants used in the bioassays of test T0b were 8 weeks old instead of 14–17 weeks as for tests T0a, T1a and T1b. Plant age has been reported to affect the resistance level to *P. infestans* (HODGSON 1961). The loci *Pi0(b)–Pi0(e)* might, therefore, indicate the existence of QTLs for resistance depending, to some degree, on plant age.

Relationships between positions of QTLs for *P. infestans*, *R* loci and loci involved in defense response: A QTL effect was detected in both parents in the interval formed by markers *GP21* and *GP179* on chromosome *V* (*Pi01(b)* and *Pi0(d)*, Figure 4). To the same interval, the dominant, race-specific allele *R1* has been mapped previously (LEONARDS-SCHIPPERS *et al.* 1992). Finding map positions for quantitative as well as qualitative resistance within the same short chromosome segment opens the possibility that the QTL and the *R* allele map at the same genetic locus. This hypothesis agrees well with the proposal of ROBERTSON (1985) suggesting that qualitative mutant phenotypes are the extreme allelic variants at a quantitative trait locus; a view which was supported by the experiments of BEAVIS *et al.* (1991) and EDWARDS *et al.* (1992) which associated QTLs for plant height in maize with Mendelian loci affecting the same trait.

Two dominant alleles for extreme resistance (*Rx*) to potato virus X also map at loci genetically linked to QTLs for resistance to *P. infestans*. *Rx2* has been mapped close to *GP21* on chromosome *V* and, therefore, close to *R1* whereas *Rx1* occupies a position on chromosome

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XII, 3 cM distal to *CP60* (Ritter *et al.* 1991; Leonards-Schippers *et al.* 1992). Both map positions coincide closely with chromosome segments carrying the QTLs *Pi01(b)*, *Pi0(d)* (chromosome *V*) and *Pi0(a)* (chromosome *XII*). The hypothesis that dominant alleles conferring resistance to different pathogens encode gene products of the same or of clusters of evolutionary related loci (Leonards-Schippers *et al.* 1992) may, therefore, be extended to alleles contributing to quantitative resistance.

Mapping to the same chromosome segments quantitative trait loci as well as specific functional genes involved in the disease response may indicate that those specific functional genes participate in the quantitative resistance response. Several associations of this type were identified in our QTL mapping experiment: The two loci *PC116* on chromosome *IV* and *prp1* (≡*pI471*) on chromosome *IX* (Figure 4 and Gebhardt *et al.* 1991) encode genes that are differentially expressed after infection of potato leaves with *P. infestans* (Taylor *et al.* 1990). The PRP1 protein is coded by a small gene family (Taylor *et al.* 1990) and shows very similar induction kinetics of its mRNA as phenylalanine ammonia-lyase (PAL) and 4-coumarate CoA ligase (4 CL) both of which are enzymes of the general phenylpropanoid metabolism (Fritzemeier *et al.* 1987). A group of *PAL* loci is also located on chromosome *IX* in the segment bordered by loci *GP190* and *prp1* (Gebhardt *et al.* 1991). To the same segment the minor QT locus *Pi0(c)* was allocated. *PC116* on chromosome *IV* is localized within the highly significant chromosome segment in which both parents contributed QTL effects for Phytophthora resistance (*Pi1(a)*, *Pi1(b)*, *Pi1(c)*, *Pi1(g)*, *Pi0(g)*). The genetic locus *4CL* mapping *ca.* 10 cM distal to *GP226* on chromosome *III* (*4CL(a)*; Gebhardt *et al.* 1991) coincided with the region harboring the QT locus *Pi1(d)*.

Pathogenesis related genes like *prp1*, *PAL* and *4CL* were found to be topographically related with loci conferring quantitative resistance to *P. infestans*. The kinetics of their induction varies quantitatively after infection with the fungus (Fritzemeier *et al.* 1987; Taylor *et al.* 1990). Based on these observations, it seems conceivable that allelic variants of *prp1*, *PAL* and *4CL* genes can cause differences in the quantitative resistance response to *P. infestans*.

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ORIGINAL PAPER

Natural DNA variation at candidate loci is associated with potato chip color, tuber starch content, yield and starch yield

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Abstract Complex characters of plants such as starch and sugar content of seeds, fruits, tubers and roots are controlled by multiple genetic and environmental factors. Understanding their molecular basis will facilitate diagnosis and combination of superior alleles in crop improvement programs ("precision breeding"). Association genetics based on candidate genes is one approach toward this goal. Tetraploid potato varieties and breeding clones related by descent were evaluated for 2 years for chip quality before and after cold storage, tuber starch content, yield and starch yield. Chip quality is inversely correlated with tuber sugar content. A total of 36 loci on 11 potato chromosomes were evaluated for natural DNA variation in 243 individuals. These loci included microsatellites and genes coding for enzymes that function in carbohydrate metabolism or transport (candidate loci). The markers were used to analyze population structure and were tested for association with the tuber quality traits. Highly significant and robust associations of markers with 1–4 traits were identified. Most frequent were associations with chip quality and tuber starch content. Alleles increasing tuber starch content improved chip quality and vice versa. With two exceptions, the most significant and robust associations ($q < 0.01$) were observed with DNA variants in genes encoding enzymes that function in starch and sugar metabolism or transport. Comparing linkage and linkage disequilibrium between loci provided evidence for the existence of large haplotype blocks in the breeding materials analyzed.

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Introduction

Most characters important for crop quality show quantitative phenotypic variation, due to the fact that they are controlled by natural DNA variation at multiple loci and by environmental factors. Knowing the molecular basis of the genetic components of this variation will facilitate the selection of improved cultivars with DNA-based markers, which are diagnostic for superior alleles of the underlying genes.

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Genetic dissection of plant complex traits in quantitative trait loci (QTL) first became possible with the advent of DNA-based markers (Osborn et al. 1987). Since then, the first genes and their allelic variants underlying plant QTL have been identified by positional cloning (reviewed in Salvi and Tuberosa 2005). Positional QTL cloning requires the generation and analysis of large experimental mapping populations. This is a labor and time-intensive process, which is feasible in inbreeding species with short generation time such as Arabidopsis, rice or tomato, but is rather prohibitive in polyploid, non-inbred species such as potato or in species with long generation time such as trees. An alternative to positional QTL cloning is the candidate gene approach, which is based on the knowledge of a gene's function in controlling a character of interest on the one hand, and genetic co-localization of a functional candidate gene with QTL for the character of interest on the other (Pflieger et al. 2001). DNA variation of genes fulfilling these criteria is examined in natural populations of individuals related by descent for associations with positive or negative character values (Li et al. 2005; González-Martínez et al. 2007). Finding such associations indicates that DNA variation either at the candidate locus itself or at a physically linked locus is causal for the phenotypic variation. Indirect associations due to physical linkage depend on the extent of the linkage disequilibrium (LD) present in the population under study. Functional complementation analysis using candidate gene alleles provides direct evidence that a candidate gene underlies a QTL (Salvi and Tuberosa 2005). Association mapping can produce diagnostic markers that are useful for plant breeding, irrespective of whether the association is direct or indirect.

Quantitative traits linked to carbohydrate contents can serve as model for the candidate gene approach to QTL identification in crops. Carbohydrates are the primary products of photosynthetic CO_2 fixation and major constituents of wood and storage compounds. Starch and sugars deposited in seeds, roots, tubers, fruits or berries are the basis of human and animal nutrition. Carbohydrate metabolism has been intensively studied in plants at the physiological, biochemical and molecular level. The enzymes catalyzing the principal anabolic and katabolic reactions are ubiquitous in plants, and the coding genes have been cloned and characterized in several species (reviewed in Beck and Ziegler 1989; Frommer and Sonnewald 1995; Winter and Huber 2000; Salerno and Curatti 2003). Functional candidate genes are therefore available in abundance.

In the potato (*Solanum tuberosum*), complex traits linked to carbohydrate composition are the starch and sugar content of the tubers, which are together with tuber yield important determinants of tuber quality. With 10–25% of the fresh weight, starch is the major storage compound of the tuber. The sugars sucrose, glucose and fructose are minor tuber constituents that do not have a role as storage compounds. Glucose and fructose accumulate, however, in tubers in response to low temperature exposure, a phenomenon called "cold sweetening" (Burton 1969; Isherwood 1973). The tuber content of the reducing sugars fructose and glucose determines the color of deep fried products such as potato chips and French fries, which is the result of a Maillard reaction between amino acids and reducing sugars at high temperatures. High reducing sugar content results in dark chip color due to polyphenol formation (Habib and Brown 1957; Townsend and Hope 1960). As this is an unwanted character, potato variety development aims at selecting genotypes with low reducing sugar content during tuber storage, preferably at low temperature to prevent sprouting.

On the one hand, QTL for tuber starch and sugar content have been mapped to potato chromosomes in experimental, diploid populations (Schäfer-Pregl et al. 1998; Menendez et al. 2002). On the other hand, cloned and characterized genes of potato have also been mapped, which function in starch biosynthesis (e.g., ADP-glucose pyrophosphorylase, starch synthases, branching enzyme), starch degradation (e.g., starch phosphorylases, debranching enzyme, α-amylase), sucrose metabolism (e.g., sucrose phosphate synthase, sucrose synthases, invertases) or transport (e.g., sucrose transporters) (Chen et al. 2001; Menendez et al. 2002). Genes functional in starch and sugar metabolism, which co-localized with QTL for tuber starch and sugar content were selected as marker loci for association studies in populations of tetraploid potato individuals related by descent. A first association has been found between chip color and natural DNA variation at the invertase locus *invGE/GF* on potato chromosome IX (Li et al. 2005) that co-localized with a QTL for tuber sugar content (Menendez et al. 2002). Invertase converts sucrose in the reducing sugars glucose and fructose. Invertase encoding genes are therefore obvious functional candidates. The *invGE/GF* locus is a candidate for one of several QTL for tuber starch and sugar content, which have been localized on each of the 12 potato chromosomes (Schäfer-Pregl et al. 1998; Menendez et al. 2002; Gebhardt et al. 2005). To further dissect the molecular basis of these complex traits and to obtain diagnostic markers for breeding, we conducted an association study based on DNA variation at candidate loci in a population of tetraploid genotypes used for potato variety development.

Materials and methods

Plant material

Three populations of 100 tetraploid breeding clones each (SAR, BNA, NOR) and 36 varieties as standards were sampled from the breeding programs for chips, starch and

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Table 1 Design of the field experiments: numbers of genotypes grown, years and locations

	2002	2002	2003	2003	2003	2004
Samples	Windeby	Groß-Lüsewitz	Windeby	Groß-Lüsewitz	Ebstorf	Ebstorf
SAR	100		100			
NOR		100		100		
BNA					100	100
Standards	36	36	36	36	36	36

table potatoes of the companies SAKA-RAGIS Pflanzenzucht (SAR clones), Böhm-Nordkartoffel Agrarproduktion (BNA clones) and NORIKA (NOR clones). The genotypes resulted from a number of crosses among different varieties and breeding clones and were selected to represent the variation for chip quality, tuber yield, starch content and starch yield present in advanced commercial breeding materials in Germany. The standard varieties were: Albatros, Apart, Artis, Aula, Christa, Diana, Eldena, Fasan, Goldika, Ilona, Karlena, Kolibri, Leyla, Likaria, Marabel, Marlen, Melina, Milva, Molli, Novara, Orlando, Panda, Pirol, Ponto, Satina, Saturna, Sempra, Sirius, Solara, Solist, Terra, Theresa, Tomensa, Valisa, Velox and Vitara. Historical pedigree information for most of the varieties is available at http://potatodbase.dpw.wau.nl/potatopedigree (van Berloo et al. 2007). According to this, the standard varieties included three pairs of half sibs (Karlena and Likaria, Marabel and Milva, Satina and Velox), one pair of full sibs (Artis and Sempra) and three varieties, which were parent of another variety in the set (Saturna is parent of Marlen, Solara of Vitara and Panda of Artis, Sempra and Sirius). The pedigree structure of varieties and breeding clones used for developing new varieties is similar. A total of 20 plants per plot were propagated in the field in two consecutive years under standard phytosanitary regimes. The sample populations SAR, BNA and NOR were grown and evaluated at the breeding stations of Saka-Ragis Pflanzenzucht GbR, BNA Zuchtgesellschaft mbH and NORIKA Ltd in Windeby, Ebstorf and Groß-Lüsewitz (Germany), respectively. The standards were grown and evaluated at all three locations (Table 1). Balanced phenotypic data from 2 years were obtained for 259 genotypes (36 standards, 100 SAR and BNA breeding clones each, 23 NOR breeding clones). Based on genetic similarity analysis among the 259 genotypes (Li et al. 2005), 16 highly similar pairs of individuals were identified. One individual of each similar pair was therefore removed from the sample populations. The remaining 243 genotypes (34 standards, 90 BNA, 96 SAR and 23 NOR) constituted the population ALL.

Phenotyping

Chip quality was assessed by visually scoring the chip color after deep frying of 1.2–2.0 mm tuber slices in oil at 160–180°C for 2–3 min (Putz 1989), using a 1–9 color scale (1 = very dark chip color, very bad chip quality; 9 = very light yellow chip color, very good chip quality), first time after harvest in autumn (CQA) and second time after 3–4 months storage at 4°C (CQS). After cold storage, the average chip quality decreased. To further differentiate chip quality at the lower end of the scale, chip scores for the SAR population were extended to include negative values. Tuber starch content (TSC, percent fresh weight) was determined by specific gravity (Von Scheele et al. 1937). Tuber yield (TY, dt/ha = deciton per hectare, 1 dt = 100 kg) was determined by the tuber weight. Tuber starch yield (TSY, dt/ha) is the product of TSC × TY.

Genotyping

Genomic DNA was isolated from freeze-dried leaf tissue as described (Li et al. 2005). DNA fragments were amplified by PCR (polymerase chain reaction) according to Li et al. (2005) using primers and annealing temperatures specified in Table 2, and adjusting the extension time to amplicon length (from 30 s to 2 min). DNA polymorphisms in the amplicons were detected by SSCP (single strand conformation polymorphism) analysis as described (Li et al. 2005), or by agarose gel electrophoresis of amplicons with (CAPS = cleaved amplified polymorphic sequence) or without (SCAR = sequence characterized amplified region, ASA = allele specific amplification) restriction enzyme digestion. SSR (simple sequence repeat) alleles were separated on Spreadex gels (Elchrom Scientific, CH-6330 Cham, Switzerland) according to the supplier's instructions. DNA fragments of all marker types were recorded in each individual as 1 for presence, 0 for absence or as missing value in unclear cases.

Data analysis

Analysis of genetic similarity was done as described (Li et al. 2005), based on 182 DNA fragments. Population substructure was evaluated using the software STRUCTURE (Pritchard et al. 2000). The dominant markers were coded into sets of four haplotypes, to correctly represent the tetraploid individuals. Within STRUCTURE, we chose to represent DNA fragments when present by

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Table 2 Locus and marker information

Locus (accession number or reference)	Encoded protein	Chromosome no.	Primer name	Primer sequence (5′–3′) or reference	Transcript size (bp)	PCR product size (bp)	No. of DNA fragments scored (assay type)	Annealing temperature (°C)
STM0038	Non-coding SSR	II	STM0038	Milbourne et al. (1998)	–	80–110	6 (SSR)	54
G6pdh (X74421)	Glucose-6-phosphate dehydrogenase	II	G6pdh-4	f-cac aat gaa aga tgg gaa gg r-ttg cac ata ggc agg gta ct	585	2000	3 (SSCP)	58
*Stp*23 (D00520)	α-Glucan phosphorylase	III	Stp23-7	f-ctg ttg tcc cag atg caa tg r-tcc ttc caa cga tcc aat ta	632	>2000	3 (SSCP)	56
			Stp23-8	f-gca aca gct caa agt gtt cg r-cac ctc ctc ctg acc atc tt	375	698	2 (SSCP)	57
Pain1 (X70368)	Soluble acid invertase	III	Pain1-5	f-cgg aat tgg att gtg gaa ttg r-tgg cgt tag ctc aga tag ctt	494	900	4 (SSCP)	58
			Pain1-8	f-gcc gtc aag agg tgt ttc tc r-acc cag tcc aga cac cgt ta	335	2000	4 (SSCP)	60
			Pain1-9	f- gat caa aag cta tct gag cta acg r- aag ctc tcc aca att gag tgg t	209	450	1 (SSCP)	61
*Sss*I (Y10416)	Soluble starch synthase I	III	SssI-4	f-gca gga tgc gac ata cta ttg r-tcc act ctt ctc cca aag ga	594	2000	2 (SSCP)	58
			SssI-11	f-tgg tgg att agg aga tgt ttg r-aag agt ggt cca caa ata ccc	233	900	3 (SSCP)	58
			SssI-7	f-cta cat gag ctg ttg agc agt ag r-cca atc aga gga caa tca gg	200	1031	1 (SSCP)	60
StKI (AF459077)	Kunitz-type inhibitor, putative invertase inhibitor	III	inhia	f-gtg ttt gct tcc cat ttt g r-cta cac cca ctt ttg cac ag	560	560	4 (SSCP)	57
			inhib	f-gtg ttt ggt tcc cat tgt gg r-agc agg cag aca gac att tg	639	639	4 (SSCP)	57
Fbp-cy (X76946)	Fructose-1,6-bisphosphatase, cytoplasmic	IV	Fbp-cy-1	f-tgc agg gag aag atc aaa aga aac r-tga aga acc atc agc ggg ata c	520	1500	1 (CAPS/RsaI)	57
AmyZ (M79328)	α-Amylase	IV	Amy-3	f-gct aag atc ctc tgt tgg ctt c r-ctt gat tcc tcc gaa tag ca	533	2000	1 (SSCP)	57
StpL (X73684)	L-type starch phosphorylase	V	StpL-3	f-gtt cag aga cat cat ggc aac r-agt cca gaa agc aag aag ca	614	1300	7 (SSCP)	58
Sut2 (AY291289)	Putative sucrose sensor	V	Sut2-3	f-gtg tca gag aag gtg cat ttg r-caa cca aaa tgg aag cca gt	547	1200	2 (SSCP)	57
			Sut2-5	f-ggc tat gcg gtc cta tta ctg r-caa gat cga gca tcc aaa ac	887	887	2 (SSCP)	58

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Table 2 continued

Locus (accession number or reference)	Encoded protein	Chromosome no.	Primer name	Primer sequence (5′–3′) or reference	Transcript size (bp)	PCR product size (bp)	No. of DNA fragments scored (assay type)	Annealing temperature (°C)
			Sut2-6	f-gtt ttg gat gct cga tct tg r-gag att tcc aca tgg ctc ac	650	690	2 (SSCP)	58
			Sut2-7	f-gtt gtg agc cat gtg gaa atc r-gtg aca gcg gga ctt cat ta	977	800 + 1300	3 (SSCP)	60
			Sut2-8	f-atg cat tcg gtt ctc att gtc r-cca aag cag caa ttt gag tt	689	689	1 (SSCP)	56
			Sut2-9	f-ctt ggc att cct ctt gct g r-atg gaa gcc agt tga ttt ga	623	690	3 (SSCP)	56
			Sut2-11	f-agc gtg gtt tct atc agt gc r-ctt cag taa cat gaa cta cat gtg ta	788	800	3 (SSCP)	58
			Sut2-12	f-aga agc tcc gtt gct tat tca r-tat tag caa gat cga gca taa a	455	455	1 (SSCP)	55
GP79 (AJ492261)	Genomic DNA fragment	VI	GP79	f-gtc ttt agg gat atg gat tac r-cct tta ctt gta att atg cat c	–	1500	1 (CAPS/Vsp509I)	53
Sps (X73477)	Sucrose phosphate synthase	VII	Sps-3	f-agc att tgg tga atg tcg tc r-gtt gcc ttg gtt tgc agc ta	530	1500	8 (SSCP)	58
			Sps-7	f-gaa aga ggt cgc aga gaa gca g r-cga tat aca cct ggc atc g	314	800	5 (SSCP)	60
			Sps-15	f-ggt cac tca ctt ggt aga ga r-tct ttg cag caa gac ggt ag	691	1600 + 500	2 (SSCP)	57
Sus3 (U24088)	Sucrose synthase 3	VII	Sus3-1	f-cat gac aag gaa agc atg acc cc r-gca aag taa atc tta tac atg tga cc	1215	1215	4 (SSCP)	57
			STM1043	Milbourne et al. (1998)	–	210–230	4 (SSR)	53
			STM1097	Milbourne et al. (1998)	–	80–130	6 (SSR)	54
Pha2 (X76535)	Plasma membrane H^+ -ATPase 2	VII	Pha2-3	f-gct gag acc atc cgt aga gc r-agg atg gca atg atc aaa ac	566	900	5 (SSCP)	56
AGPaseB (X61186)	ADP-glucose pyrophosphorylase B	VII	AGPsb-1	f-gat tct aca cgt gct gta tcc ag r-ggc aga gtt gaa ttg tgt ga	378	1500	1 (SSCP)	56
			AGPsb-2	f-tcc tgt aag caa ctg ctt gaa c r-tca tga gac caa atg cag tg	383	800	1 (SSCP)	56
			AGPsb-6	f-gca act tca ctt ggg atg ag r-caa gca ttt ttg atg gtg gt	190	700	4 (SSCP)	54
			AGPsb-15	f-gtc aca gat agt gtc att ggt ga r-tgc atg atc tga gtc caa cc	81	800 + 1000	3 (SSCP)	60

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Table 2 continued

Locus (accession number or reference)	Encoded protein	Chromosome no.	Primer name	Primer sequence (5′–3′) or reference	Transcript size (bp)	PCR product size (bp)	No. of DNA fragments scored (assay type)	Annealing temperature (°C)
Pat (X04077)	Patatin	VIII	STM1055	Milbourne et al. (1998)	–	200–230	1 (SSR)	53
GP171 (CG783103)	Genomic DNA fragment	VIII	GP171	f-ctg cag ctt ttc tct tgt ctg r-tgc agt ccg aat aac tgt ga	–	400 + 600	1 (SCAR)	58
GbssI (X52417)	Granule-bound starch synthase I	VIII	STM1104	Milbourne et al. (1998)	–	160–200	7 (SSR)	57
STM1052	Non-coding SSR	IX	STM1052	Milbourne et al. (1998)	–	210–250	3 (SSR)	50
Inv-ap-b (AJ133765)	Invertase, apoplastic	IX	InvGE-6	Li et al. (2005)	432	432 + 367	4 (SSCP)	59
			InvGF-4d	Li et al. (2005)	–	248	1 (ASA)	63
			InvGF-4b	Li et al. (2005)	–	296	1 (ASA)	61
STM3012	Non-coding SSR	IX	STM3012	Milbourne et al. (1998)	–	160 – 200	4 (SSR)	57
StpH (Mori et al. 1991)	H-type starch phosphorylase	IX	StpH-3	f-gaa gga ctt ggg tgg gat g r-caa act ccc gaa gat tag ca	511	1500	3 (SSCP)	57
			StpH-13	f-gta tct gtg gca gag atg ctt r-agc atc cat gta gct cgg aaa	404	1500	3 (SSCP)	59
STM3023b	Non-coding SSR	IX	STM3023b	Milbourne et al. (1998)	–	180–200	3 (SSR)	50
STM2012	Non-coding SSR	X	STM2012	Milbourne et al. (1998)	–	240 – 260	2 (SSR)	64
Rca (J03610, AF037361, X14212, Z21794)	Ribulose bisphosphate carboxylase activase	X	Rca-1	f-aca ccg tca aca acc aga tg r-act ctc ttg aca ttc tct tgc	502	600 + 300	1 (SCAR)	56
			Rca-3	f-ccc ttg aga agc tcc ttg ag r-ttc cct taa cag tgg aac aca a	333	450	3 (SSCP)	57
Inv-ap-a (Z22645)	Invertase, apoplastic	X	pCD141-3	f-aca agt ttg gat aag gca gag r-tag agt ctc aat tgt gat tct ctc c	459	700	3 (SSCP)	55
STM1106	Non-coding SSR	X	STM1106	Milbourne et al. (1998)	–	120–190	9 (SSR)	60
CT120	Tomato cDNA	XI	CT120	Huang et al. (2004)		360	2 (CAPS/RsaI) 1 (CAPS/TaqI) 2 (CAPS/Tsp590I)	52
GP250 (CG783183)	Genomic DNA fragment	XI	GP250	Huang et al. (2004)	–	410	1 (CAPS/VspI)	52
St1.1 (U60069)	Genomic DNA fragment, resistance gene like	XI	St1.1	Huang et al. (2004)	–	450	2 (CAPS/HaeIII) 1 (CAPS/HinfI)	52
cLET5E4 (AW038480)	Tomato cDNA	XI	cLET5E4	Huang et al. (2004)	331	310	2 (CAPS/Tsp590I)	55
Sut1 (X69165)	Sucrose transporter 1	XI	Sut1-7	f-gta atc gtg gtt cag gac aag r-acc cca ttt gtt tgg aag aa	443	1300	2 (SSCP)	53

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Table 2 continued

Locus (accession number or reference)	Encoded protein	Chromosome no.	Primer name	Primer sequence (5′–3′) or reference	Transcript size (bp)	PCR product size (bp)	No. of DNA fragments scored (assay type)	Annealing temperature (°C)
Dbe (A52190)	Debranching enzyme	XI	Dbe-5	f-gtg acc cta ctg tgt ctc atg aa r-ttt gga act ccc cag aga ac	301	1300	7 (SSCP)	57
			Dbe-8	f-ttg ctg gtg tcg aag atg ag r-tcc tca gaa tct ggt gga aa	82	450	1 (SSCP)	60
STM0037	Non-coding SSR	XI	STM0037	Milbourne et al. (1998)	–	70–90	7 (SSR)	48
Ppa1 (Z36894)	Soluble inorganic pyrophosphatase	VIII, XII	Ppa1-2	f-tga tcg cat cct ata ctc ttc ag r-aga gca gca agg gat aaa cc	595	1200	2 (SSCP)	58
Sus4 (U24087)	Sucrose synthase 4	XII	Sus4-2	f-ctc atg gat att ttg ccc aag r-ttt ccg ttt cgg agt acg ag	1209	1209	2 (SSCP)	58

SSR simple sequence repeat, *SSCP* single strand conformation polymorphism, *CAPS* cleaved amplified polymorphic sequence, *SCAR* sequence characterized amplified sequence, *ASA* allele specific amplification

(1, *, *, *) for each individual, because of the dominant scoring, whereas absence was coded by (0, 0, 0, 0).We chose a model with admixture and independent allele frequencies. The number of subpopulations (K) was set to vary between 2 and 30. For each fixed K, two independent MCMCs (Markov Chain Monte Carlo) were run using 600,000 iterations for each. The first 100,000 iterations were discarded as burn-in. The likelihood of the data given K was saved for each K value. The underlying number of subpopulations in the sample was estimated by the K value that had the highest likelihood.

LD between all pairs of DNA fragments was calculated using Fisher's exact test on two-by-two contingency tables (DNA fragment present or absent), using GenStat (GENSTAT 2005). The P values were corrected for multiple testing to correspond to a false discovery rate (FDR) equal or smaller than 0.05 (see below). DNA fragments with a frequency (fraction of individuals having the fragment) less than 10% or higher than 90% were excluded from the association analysis.

Association analysis was carried out using various regression and mixed models in GenStat (GENSTAT 2005). First, the adjusted means of each trait were extracted in a model that corrects the observations for the conditions of a specific trial (company performing the trial and year of the trial):

$$y = \text{trial} + \text{genotype} + \text{error}.$$

The factor *trial* had six levels describing differences in environmental conditions related to location, soil type and scoring rules. Each level of *trial* corresponded to the measurements performed by the same company and in the same year (Table 1). Genotype is a factor that identifies breeding clones or varieties as measured in the different trials. Adjusted genotypic means from the model above were used as response variable in the next model that tests for marker-trait associations:

$$y^* = \text{origin} + \text{marker} + \text{error} \quad (1)$$

where y^* stands for adjusted means saved from the previous model. *Origin* is a factor with four classes to identify the origin of each genotype in the sample: three corresponding to the genotype groups "SAR", "BNA" and "NOR", and the fourth to the group "Standards". Marker is a factor with two levels, for DNA fragment present or absent. Its association with the trait was tested by a (partial) t test, testing the additional variation explained by a marker after correction for origin. The resulting P values were converted into q values (see below). The proportion of genetic variation explained by each marker was calculated as the relative increase in R^2 when the marker is added to the model. Finally, we constructed multi-QTL models by applying stepwise

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selection to the set of significant markers found in the previous single-marker regression model (1):

$$y^* = \text{origin} + \sum \text{markers} + \text{error}$$

To identify multi-QTL models, we used an F value for entering the regression model, $F_{in} = 4$, and an F value for dropping out of the model, $F_{out} = 4$ (Montgomery and Peck 1982).

In addition, we investigated the influence of possible population substructure, by fitting two mixed models, (2) and (3), that were more complex than model (1) and attempted to correct for population substructure in more sophisticated ways, as described by Yu et al. (2006) and Malosetti et al. (2007). The mixed model (2) included a marker-based genetic similarity matrix to account for kinship between genotypes:

$$y^* = \text{marker} + \text{genotype} + \text{error} \quad (2)$$

with fixed marker and random genotype. The covariance between two genotypes i and j is given by $\theta_{ij}.\sigma_g^2$ with σ_g^2 being the genetic variance, and θ_{ij} the pairwise genetic similarity coefficients calculated as Jaccard indices based on DNA information. Mixed model (3) corrected for both kinship and origin. For comparison, association analysis was also performed without correcting for kinship and origin (model 4).

The statistical tests for LD between DNA fragments and the association analysis in the population ALL were corrected to control the false discovery rate (FDR). The FDR is the expected proportion of false associations in the total set of significant associations. The P values were adjusted according to the Two-Stage Linear Step-Up Procedure (Benjamini et al. 2005), from which q values were obtained. These q values provide each test with an individual measure of significance in terms of an FDR of 0.05 (5% false associations in the total set of significant associations).

Genetic mapping

The diploid population H94A was used for mapping, where linkage maps have been constructed for all chromosomes based on AFLP (amplified fragment length polymorphism) and RFLP (restriction fragment length polymorphism) markers (Menendez et al. 2002). The same primers as used for association mapping were used to amplify markers from DNA of the parents and 150 F1 individuals of the H94A population. The amplicons were analyzed for SSCPs. Segregating SSCP alleles were scored as present or absent and tested for linkage with AFLP or RFLP markers of known map position. Genetic distance in centimorgan between linked marker loci was estimated as described (Menendez et al. 2002).

Results

Populations and phenotypic data

Chip quality after harvest in autumn (CQA) and in spring after cold storage (CQS), tuber yield (TY), tuber starch content (TSC) and tuber starch yield (TSY) were evaluated in the tetraploid breeding clones of Saka-Ragis (SAR), Böhm-Nordkartoffel Agrarproduktion (BNA) and NORIKA (NOR) and in the standard varieties (Table 1). The adjusted genotypic means of each trait in the sub-populations BNA, SAR, NOR and Standards, and in the ALL population (the combination of SAR, BNA, NOR clones and standard varieties after removal of potential duplicates) are shown in Fig. 1. Chip quality was generally lower after storage at 4°C, due to the accumulation of reducing sugars at low temperature (cold sweetening). The analysis of residuals, resulting from the association models (see below), showed normal distribution and independence for all traits, and the variance was homogeneous (not shown).

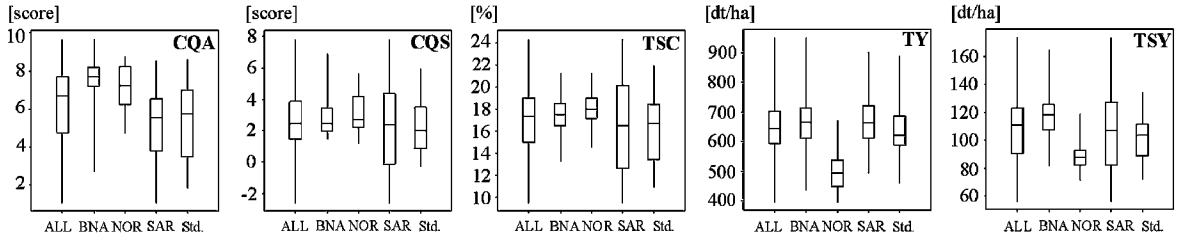


Fig. 1 Box plots of the adjusted genotypic means of 2 years of traits *CQA* (chip quality autumn), *CQS* (chip quality spring), *TSC* (tuber starch content), *TY* (tuber yield) and *TSY* (tuber starch yield) in sample populations *BNA* (90 individuals), *NOR* (23 individuals), *SAR* (96 individuals) and Standards (*Std.*, 34 individuals), and in the *ALL* population (243 individuals). Based on genetic similarity 16 individuals were excluded from the populations . The boxes span the interquartile range of the trait values, so that the middle 50% of the data lay within the box, with a line indicating the median. Whiskers extend beyond the ends of the box as far as the minimum and maximum values. Chip quality was scored from 1 to 9, score 1 corresponding to bad chip quality. To evaluate chip quality after cold storage with a similar range, the scale was extended to scores <1, resulting in whiskers extending into negative values in the CQS box plot. Tuber starch content is given in % (w/w), and yield and starch yield in dt/ha (1 dt = 100 kg)

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Genotypic data and population structure

The SAR, BNA and NOR individuals and the standards were scored for 188 polymorphic DNA fragments generated at 36 loci on all potato chromosomes except chromosome I (Fig. 2, Table 2). From 1–17 polymorphic DNA fragments were scored at each locus, derived from 1–8 amplicons generated with different primer pairs, which annealed to various regions of a gene's sequence (Table 2). A total of 23 loci encoded genes with known function, mostly in carbohydrate metabolism (Table 3). The remaining 13 loci were genomic fragments of unknown coding capacity or gene fragments of unknown function. The DNA fragments were used to analyze genetic similarity between the individuals and to evaluate population substructure (Pritchard et al. 2000). We did not find evidence for population substructure in the ALL population. The likelihood of our sample kept increasing with K for all tested values of K (Fig. 3), suggesting no obvious structure in the analyzed individuals.

Linkage and linkage disequilibrium (LD) between markers

LD was estimated for all pairs of DNA fragments (Fig. 4). Highest LD values were observed between DNA fragments originating from the same locus, e.g., from different regions of the same gene. One locus corresponded to approximately 500–8,000 base pairs genomic DNA sequence. In a number of cases, DNA fragments generated from different regions of the same gene showed very high LD to each other, due to the fact that the same allele was detected by more than one DNA fragment (e.g., *Stp23* and *Pain1*; Table 4). Some DNA fragments were in high LD because they appeared mutually exclusive. Despite similar fragment frequencies, individuals heterozygous for both DNA fragments were very rare or absent in the population (not shown). Examples for mutually exclusive alleles are *Sut2-7c* and *Sut2-7b* on chromosome V, both associated with tuber starch content but with opposite effect (Supplementary Table 2). This observation violates the assumption that the alleles combine at random in the population and genotype frequencies are determined only by the allele frequencies (Hardy-Weinberg equilibrium). We cannot rule out the possibility, however, that the mutually exclusive alleles resulted from preferential PCR amplification of one allele versus the other in heterozygous individuals.

LD was also observed among DNA fragments at physically linked loci on the same chromosome and with low frequency between unlinked loci on different chromosomes (Fig. 4). The extent of LD was assessed by estimating the

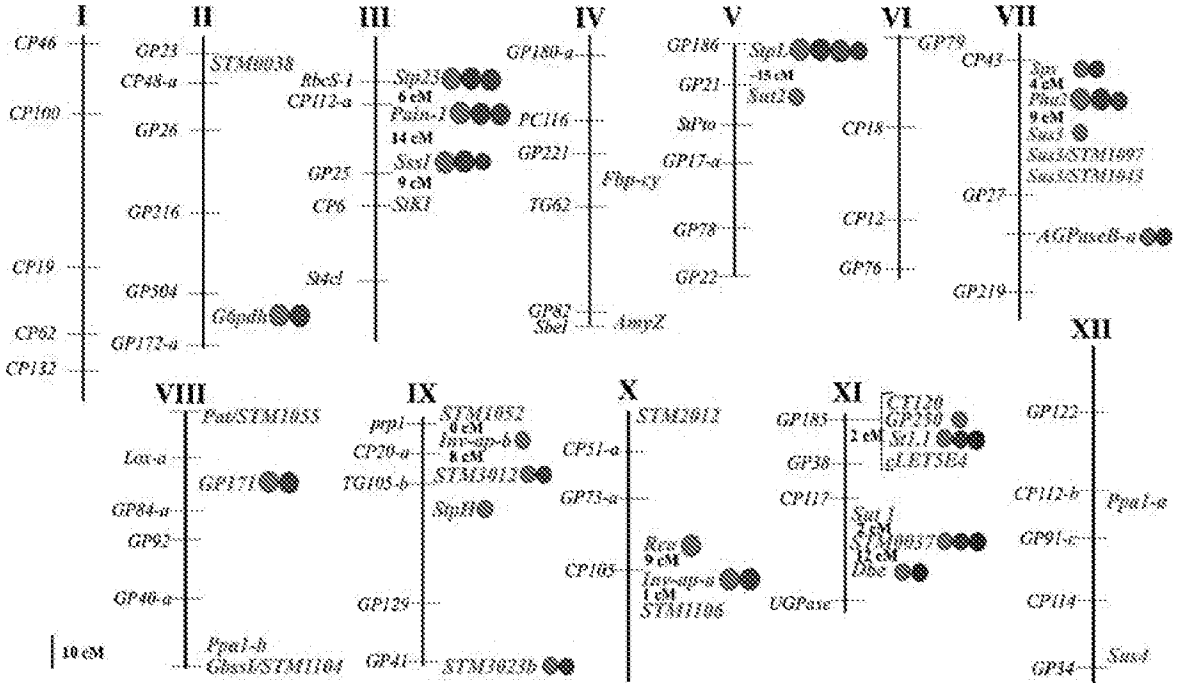


Fig. 2 Map positions of genotyped loci and marker-trait associations. Potato chromosomes are represented by 12 linkage groups based on genetic distances between RFLP markers shown on the left, which were mapped in reference molecular maps (Gebhardt et al. 2001, 2003; https://gabi.rzpd.de/projects/Pomamo/). The loci genotyped in the populations are shown in blue. Genetic distances are indicated in centimorgan (cM). Marker-trait associations are indicated by *orange circles* (online) for chip quality, *blue circles* (online) for tuber starch content, *red circles* (online) for tuber yield and *purple circles* (online) for starch yield. Circles with larger diameter indicate the associations with the largest effects (Table 4)

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Table 3 Functional genes tested for association with tuber quality traits

Locus	Chromosome no.	Accession number or reference	Encoded protein	Metabolic role
G6pdh	II	X74421	Glucose-6-phosphate dehydrogenase, cytosolic	Oxidative pentose phosphate pathway, provision of NADPH and sugar phosphates (Copeland and Turner 1987)
Stp23	III	D00520	α-Glucan phosphorylase, L-type, plastidic	Starch degradation
Pain1	III	X70368	Soluble acid invertase, vacuolar	Sucrose cleavage
SssI	III	Y10416	Soluble starch synthase I	Starch synthesis
StKI	III	AF459077	Kunitz-type enzyme inhibitor, putative invertase inhibitor	Enzyme inhibition, storage protein (Glaczinski et al. 2002)
Fbp-cy	IV	X76946	Fructose-1,6-bisphosphatase, cytosolic	Sucrose synthesis
AmyZ	IV	M79328	α-Amylase	Starch degradation
StpL	V	X73684	L-type starch phosphorylase, plastidic	Starch degradation
Sut2	V	AY291289	Putative sucrose sensor or transporter	Sugar transport (Barker et al. 2000)
Sps	VII	X73477	Sucrose phosphate synthase	Sucrose synthesis
Sus3	VII	U24088	Sucrose synthase 3	Sucrose conversion
Pha2	VII	X76535	Plasma membrane H^+ -ATPase 2	Driving proton coupled active sucrose transport
AGPaseB-a	VII	X61186	ADP-glucose pyrophosphorylase, B subunit	Starch synthesis
Pat	VIII	X04077	Patatin	Storage protein
GbssI	VIII	X52417	Granule-bound starch synthase I	Starch synthesis
Inv-ap-b	IX	AJ133765	Invertase, apoplastic	Sucrose cleavage
StpH	IX	Mori et al. (1991)	H-type starch phosphorylase, cytosolic	Starch degradation
Rca	X	J03610, AF037361, X14212, Z21794	Ribulose bisphosphate carboxylase activase	CO_2 fixation, Calvin cycle, photorespiration
Inv-ap-a	X	Z22645	Invertase, apoplastic	Sucrose cleavage
Sut1	XI	X69165	Sucrose transporter 1	Sucrose transport
Dbe	XI	A52190	Debranching enzyme	Starch degradation
Ppa1	VIII, XII	Z36894	Soluble inorganic pyrophosphatase	Driving carbohydrate anabolism
Sus4	XII	U24087	Sucrose synthase 4	Sucrose conversion

recombination frequency between four loci on chromosome III (*Stp23, Pain1, SssI, StKI*), three loci each on chromosomes VII (*Sps, Pha2, Sus3*), IX (*STM1052, Inv-ap-b, STM3012*), X (*Rca, Inv-ap-a, STM1106*) and XI (*Sut1, STM0037, Dbe*) by genetic mapping in a diploid experimental population. Loci *CT120, GP250, St1.1* and *cLET5E4* on chromosome XI are tightly linked within 2 cM (Huang et al. 2004). The genetic distances between these loci are shown in Fig. 2. LD was observed between DNA fragments at loci that were up to 14 cM apart (Figs. 2, 4). For example, alleles at the *Pain1* locus on chromosome III showed LD with alleles at *Stp23* located 6 cM in the distal and *SssI* located 14 cM in the proximal direction. Some alleles at loci *Sps, Pha2* and *Sus* located within 13 cM on chromosome VII were in LD with each other. This indicated the existence of large haplotype blocks in the genetic material analyzed, at least in some regions of the potato genome.

Marker-trait associations

A total of 150 DNA fragments with frequencies between 10 and 90% in the ALL population were tested for association with traits CQA, CQS, TSC, TY and TSY using four different models. All DNA fragments associated with any trait at $q < 0.05$ with any of the four models are reported in Supplementary Table 1. All models tested gave similar results concerning major marker-trait associations.

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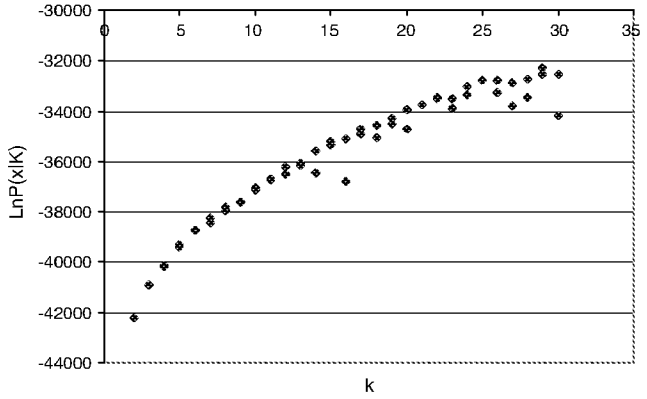


Fig. 3 Analysis of substructure in the ALL population using STRUCTURE (Pritchard et al. 2000). The number of subpopulations (*K*) was set to vary between 2 and 30. For each fixed *K*, two independent MCMCs (Markov Chain Monte Carlo) were run. The likelihood of the data given *K* (ln *P*(*X*|*K*)) is plotted for each value of *K*

The most relevant marker-trait associations obtained with the multiple regression model (1) are summerized in Table 4. Further details such as allele frequency, direction of effect and variance explained for all marker-trait associations found with model (1) ($q < 0.05$) are reported in Supplementary Table 2. A total of 66 DNA fragments (44%) were associated with one or more traits at $q < 0.05$. When DNA fragments of the same locus with highly similar distribution in the population and consequently similar associations were considered as single alleles or haplotypes, 46 alleles at 22 loci on eight chromosomes were associated with one or more traits (Supplementary Table 2; Fig. 2). Twenty-five marker alleles each were associated with CQA and CQS, respectively; twenty-seven alleles were associated with TSC, one with TY and nine with TSY. Except markers *GP171* and *STM0037*, the most significant associations ($q < 0.01$) were detected with DNA polymorphisms at eight loci, which encode the enzymes glucose-6-phosphate dehydrogenase (*G6pdh*), the starch phosphorylases *Stp23* and *StpL*, the soluble starch synthase *SssI*, the invertases *Pain1* and *Inv-ap-a*, plasma membrane H^+ -ATPase 2 (*Pha2*) and ribulose bisphosphate carboxylase activase (*Rca*) (Table 4; Supplementary Table 1).

Half of the 46 alleles were associated with more than one trait. Most frequent were associations both with chip quality and tuber starch content (Fig. 2, Table 4; Supplementary Table 2). Without exception, the direction of the effect, whether increasing or decreasing the trait mean, was the same for all alleles associated with chip quality and tuber starch content (Table 4). In contrast, the allele *StpL-3e* associated with tuber starch content and yield showed an opposite effect. Higher starch content reduced yield and vice versa (Table 4). Six loci were associated with chip quality, tuber starch content and starch yield, *Stp23*, *PainI*, *SssI*, *Pha2*, *St1.1* and *STM0037* (Fig. 2, Table 4). Alleles at the *StpL* locus were associated with all traits evaluated (Fig. 2, Table 4). Different alleles at the same locus could be associated with different effects. For example, allele *StpL-3b* decreased chip quality, starch content and starch yield, whereas allele *StpL-3e* increased chip quality and starch content but lowered yield. *StpL-3d* affected negatively only chip quality (CQS), whereas *StpL-3c* had a negative effect only on tuber starch content. At the *Pain1* locus, allele *PainI-5b* had negative, and *PainI-9a* positive effects on the associated traits. The positive association with chip quality of invertase alleles *InvGE-6f* and *InvGF-4b* at the *Inv-ap-b* locus on chromosome IX found previously in independent populations (Li et al. 2005), was again observed in the ALL population (Table 4).

Stepwise selection identified 10, 8, 8 and 6 marker alleles for CQA, CQS, TSC and TSY, respectively, which are highlighted in bold letters in Table 4. These markers collectively explained 39.7, 49.0, 54.9 and 26.1%, respectively, of the total variation in the ALL population.

Discussion

Our experiment is among the most comprehensive association studies for important agronomic traits that are currently available for crop plants, comprising association tests for multiple traits at multiple loci in advanced varieties and breeding materials (Wilson et al. 2004; Breseghello and Sorrells 2006; Wei et al. 2006; González-Martínez et al. 2007). Chip quality, tuber starch content and yield were evaluated at three breeding stations with the standard methods used for variety selection. Proprietary breeding clones were evaluated at single locations. To facilitate joint data evaluation, the same set of varieties was included in the trials at all locations. This experimental design may serve as model in association studies based on proprietary breeding materials that cannot be shared. Marker-trait associations were detected using multiple regression and mixed models, which took into account trials, locations, years, clone origin and kinship. Pedigree information as suggested (Malosetti et al. 2007) was not available for most of the breeding clones. Instead, a marker-based genetic similarity matrix was included in models 2 and 3 to account for kinship, similarly as in Yu et al. (2006). Clone origin was included in models 1 and 2, because the SAR, BNA, NOR and "Standards" populations represented four independent samples from the tetraploid germplasm adapted to potato cultivation in temperate European climate. The marker-trait associations with the largest effects were consistent across different models. The results were more variable, depending on the model, for associations with minor effects. The minor

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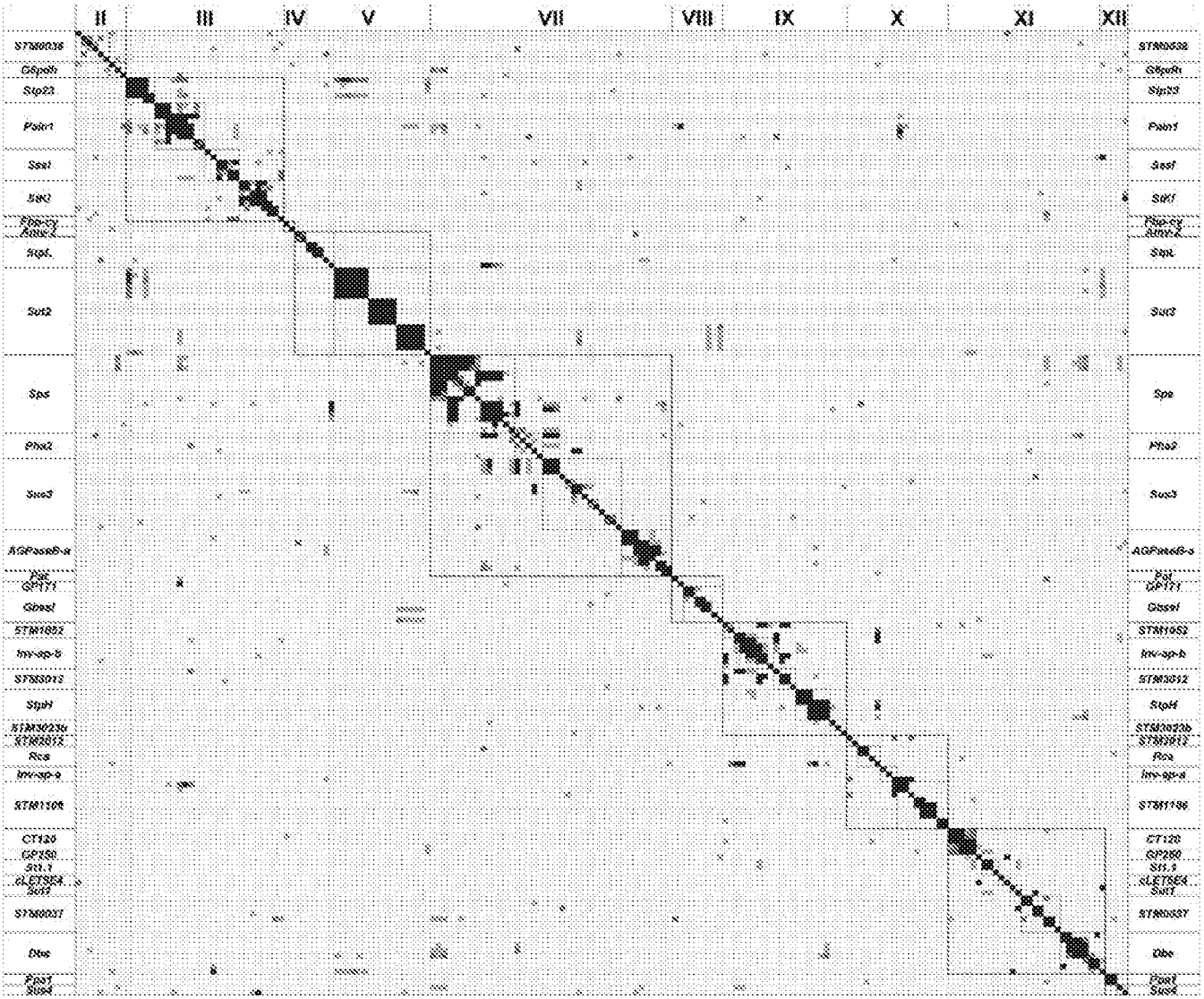


Fig. 4 LD matrix between DNA fragments in the ALL population. DNA fragments originating from the same locus are framed with *thin black lines*. The loci are arranged according to their physical order on the chromosome. Locus names are shown on the left and right of the matrix. Loci on the same chromosome (chromosome number indicated at the top) are framed with *bold black lines*. *Black cells* LD between DNA fragments is significant at $P < 0.000013$. *Dark gray cells* LD between DNA fragments is significant at $0.000013 < P < 0.00013$. *Light gray cells* LD between DNA fragments is significant at $0.00013 < P < 0.0013$. The threshold value $P < 0.0013$ was derived from the multiple testing correction (FDR ≤ 0.05)

association of invertase alleles *InvGE-6f* and *InvGF-4b* with chip quality, found in the ALL population, confirmed the previous results obtained in independent samples of breeding materials (Li et al. 2005). This provides the first evidence for the reproducibility of a marker-trait association in different samples. As observed in other studies with similar genetic materials (Li et al. 2005; Simko et al. 2006; Malosetti et al. 2007), middle European potato varieties and advanced breeding clones so far represent unstructured populations, probably due to a rather homogeneous genetic background.

At least one marker allele at 22 of the 36 examined loci showed association with one or more traits. This was an unexpected high number of associations, when considering the small proportion of the potato genome that was sampled. There are two explanations for this finding. First, allelic variation at some loci may indeed be causal for the trait variation. About two-thirds of the loci were selected based on co-localization of functional candidate genes with QTL for tuber sugar and/or starch content (Beck and Ziegler 1989; Frommer and Sonnewald 1995; Winter and Huber 2000; Salerno and Curatti 2003;

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Table 4 Most significant and relevant marker-trait associations in the ALL population

Locus	Chromosome no.	Marker allele	CQA q (R^2)	CQS q (R^2)	TSC q (R^2)	TY q (R^2)	TSY q (R^2)
G6pdh	II	*G6pdh-4d*	**0.000 (9.2)** ↓[c]	0.000 (10.4) ↓	0.000 (6.9) ↓	ns[b]	ns
Stp23	III	*Stp23-8b (7a, 7b)*[a]	0.002 (3.9) ↑	0.000 (9.0) ↑	0.000 (11.4) ↑	ns	0.002 (6.7) ↑
	III	*Stp23-8a*	ns	**0.003 (5.2)** ↑	**0.000 (9.6)** ↑	ns	**0.002 (5.9)** ↑
Pain1	III	*Pain1-5b*	ns	0.048 (2.5) ↓	0.002 (5.3) ↓	ns	ns
	III	*PainI-9a (8c, 5c)*[a]	**0.001 (4.4)** ↑	**0.000 (10.4)** ↑	**0.000 (12.0)** ↑	ns	**0.003 (5.3)** ↑
SssI	III	*SssI-4b (7a)*[a]	**0.004 (3.7)** ↑	**0.000 (6.6)** ↑	**0.000 (7.2)** ↑	ns	**0.012 (4.3)** ↑
StpL	V	*StpL-3b*	**0.000 (5.3)** ↓	**0.000 (7.3)** ↓	**0.000 (7.0)** ↓	ns	**0.033 (3.3)** ↓
	V	*StpL-3e*	0.000 (6.8) ↑	0.000 (12.6) ↑	0.000 (9.3) ↑	**0.000 (7.7)** ↓	ns
	V	*StpL-3c*	ns	0.048 (2.5) ↓	**0.000 (6.4)** ↓	ns	ns
Sps	VII	*Sps-7c (3a, 3i)*[a]	**0.022 (2.5)** ↑	ns	ns	ns	ns
Pha2	VII	*Pha2-3a*	ns	**0.001 (6.3)** ↓	0.004 (4.7) ↓	ns	ns
AGPaseB	VII	*AGPsb-6b*	ns	**0.040 (2.9)** ↓	ns	ns	ns
GP171	VIII	*GP171-a*	**0.000 (5.7)** ↓	**0.000 (7.5)** ↓	**0.003 (4.9)** ↓	ns	ns
Inv-ap-b	IX	*InvGE-6f (InvGF-4d)*[a]	**0.034 (2.2)** ↑	**0.040 (2.8)** ↑	ns	ns	ns
Rca	X	*Rca-1a*	0.000 (6.9) ↓	0.016 (3.8) ↓	ns	ns	ns
Inv-ap-a	X	*pCD141-3c*	**0.008 (3.1)** ↓	0.028 (3.1) ↓	0.007 (4.2) ↓	ns	ns
GP250	XI	*GP250-a*	**0.045 (1.8)** ↓	ns	ns	ns	ns
St1.1	XI	*St1.1_HaeIII_b (St1.1_HinfI_a)*[a]	**0.045 (1.8)** ↑	ns	**0.033 (2.7)** ↑	ns	0.048 (2.9) ↑
STM0037	XI	*STM0037-a*	0.036 (2.0) ↑	0.043 (2.7) ↑	0.002 (5.5) ↑	ns	ns
	XI	*STM0037-g*	ns	ns	**0.003 (5.1)** ↓	ns	**0.025 (3.8)** ↓
Dbe	XI	*Dbe-5c*	ns	ns	ns	ns	**0.026 (3.5)** ↓

Marker alleles identified by stepwise selection are in bold print. The amount of variance (in %) explained by the marker is given by the R^2 statistic

[a] Marker fragments, shown in parenthesis are in nearly absolute LD, have identical or highly similar distribution in the population and show similar associations

[b] Not significant, $q > 0.05$

[c] Direction of effect: ↑ the marker allele has a positive effect on the trait (better chip quality, higher tuber starch content, yield and starch yield), ↓ the marker allele has a negative effect on the trait (lower chip quality, tuber starch content, yield and starch yield)

Schäfer-Pregl et al. 1998; Chen et al. 2001; Menendez et al. 2002; Gebhardt et al. 2005). Except *GP171* and *STM0037* on chromosome VIII and XI, respectively, the most significant and robust associations were observed with candidate gene alleles. Second, LD between alleles at loci several centimorgans apart could result in indirect associations, thereby increasing the proportion of the potato genome that was actually tagged. This is indicated by associations found with genomic and SSR markers that did not encode any candidate gene. The large haplotype blocks observed in populations of advanced potato breeding clones and varieties likely result from a limited number of meiotic recombination events separating the individuals and/or selection (Gebhardt et al. 2004; Simko et al. 2006; Malosetti et al. 2007). Based on these observations, we propose that genome-wide association studies should be feasible in potato breeding materials with, on average, one marker locus per centimorgan, amounting to 500–1,000 loci in total. Large haplotype blocks are favorable for identification of diagnostic markers for breeding purposes, but are less favored when aiming at the verification of candidate genes by association mapping. However, candidate gene alleles associated with positive or negative trait values can be isolated from carrier individuals and validated by comparative complementation analysis in heterologous model systems such as yeast, as demonstrated for tomato invertase alleles (Fridman et al. 2004).

LD was also observed between unlinked markers on different chromosomes. With a threshold of 5% false positives among all significant LD pairs, a background of false positive LD pairs is expected, particularly among pairs in the lowest significance category. Interestingly, a few unlinked markers showed very strong LD. This might result from adaptive co-selection of epistatic alleles linked to these markers in the germplasm studied. Epistatic

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marker-trait associations have been identified in the ALL population (manuscript in preparation).

Most loci associated with chip quality were also associated with tuber starch content, suggesting that these complex traits are controlled, in part, by the same genes. The pleiotropic effects of individual alleles always had the same direction, either positive (more starch, better chip color, less reducing sugars) or negative (less starch, worse chip color, more reducing sugars). Starch and sugars in dormant tubers are interconvertible, and the energy for this conversion is provided by respiration. The temperature-dependent balance between starch and sugars shifts toward sugars at low temperature (Isherwood 1973). Less efficient and/or cold labile alleles of katabolic enzymes such as invertases and starch phosphorylases might shift the balance toward higher starch and lower sugar content, whereas more active and/or cold stable alleles would have the opposite effect. The *StpL* locus on chromosome V is an example where both types of alleles were observed. The molecular basis of such differences remains to be elucidated. Possibilities are the variation of enzyme activity, stability or post-translational modifications due to amino acid changes, which alter protein conformation or modification sites, or the variation in expression level due to DNA polymorphisms in cis-regulatory sequences.

Cold sweetening has been correlated with increased enzymatic activity of invertase and starch hydrolyzing enzymes, or with the cold lability of glycolytic enzymes (Pollock and Ap Rees 1975; Pressey and Shaw 1966; Zrenner et al. 1996; Cottrell et al. 1993). In our experiment, the majority of the alleles associated with chip quality after cold storage was also associated with chip quality after harvest, before the onset of cold storage. The genes controlling sugar content in the final stages of tuber development and in the dormant, cold-stored tuber may be largely identical, and low temperature was not required for differentiating their allelic variants. Alternatively, alleles specifically effective during cold storage might have escaped detection in the association test due to low frequency in the breeding material analyzed.

In conclusion, our results demonstrate that association genetics in advanced breeding populations of the potato, a polyploid, non-inbred crop, is a valuable approach toward elucidating the molecular basis of complex agronomic traits and for developing diagnostic DNA-based markers for "precision breeding" of improved varieties.

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Dissection of

the major late blight resistance cluster

on potato linkage group IV

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Dissection of

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Anoma A. Lokossou

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in the presence of the

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Dissection of the major late blight resistance cluster on potato linkage group IV

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Chapter 1

General introduction

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General introduction

Potato (*Solanum tuberosum*) history and importance

The commercial potato derived from the wild species *Solanum tuberosum* originates from the Andes in South America. Originally it was first cultivated next to the present border separating Peru and Bolivia some 8,000 years ago. The Spanish took the potato from Latin America to Europe in the 16th century. Potato was first admired for its flowers before being appreciated for its tubers and since then potato became a major carbohydrate source in human and animal diets around the world. Adaptation to long days (Brown 1990; Hawkes 1978; 1994) and generations of breeding led to a panel of potato varieties differing in taste, skin color, shape, starch content, cooking type, etc… The fast growing characteristic of potato allowed poor families to cultivate it on small plots and break the circle of poverty. Hundred millions of people around the world depend on potato to survive. Potato is grown in more than 100 countries, under temperate, subtropical and tropical conditions and ranks as the world's third most important food crop, after rice and wheat. China is now the largest potato producer followed by India, Russian Federation and USA.

Late Blight and *Phytophthora infestans*

Monoculture and the lack of genetic diversity helped the emergence and dominance of the most significant threat to potato production worldwide: the Late Blight (LB) disease, caused by the oomycete pathogen *Phytophthora infestans*, which destroys leaves, stems and tubers. One memorable event happened in the 19th century, when Ireland was cultivating a few potato varieties sharing the same genetic background which were devastated by a Late Blight epidemic. Widespread famine followed with the death of millions of people and the migration of more than a million of Irish to the USA.

Current disease control relies on multiple (up to 20) fungicide applications per season (Hanson et al. 2007) and costs associated with chemical control and yield loss amount to €5 billion globally per year (Whisson et al. 2001). Such pesticide inputs are both environmentally and economically undesirable and have a negative impact on the sustainability of potato production. To prevent spray of too large quantities of fungicides, the focus of different scientific groups is to find an efficient decision support system for sustainable cultivation of potato (Savenkov et al. 2003). The purpose is to gather information about the emergence and the progress of *Phytophtora infestans* in the field, weather forecast, cultivars planted, date of fungicide spreads and spatial epidemiology (spore release, escape,

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transport, deposition and survival), with the aim to give a prediction about the risk of regional spore dispersal and survival helping the potato growers in an online service for decision making about when, which and how much fungicide to spray (Midilis program; Simcast (Skelsey et al. 2009).

Introgression of Late Blight Resistance by traditional breeding

Early in the 20th century the potential of the wild Mexican hexaploid species *Solanum demissum* was recognized for conferring extreme levels of Late Blight resistance and hybrids with *S. tuberosum* were generated. Subsequently, 11 major dominant resistance (*R*) genes were identified, and introgressed into potato cultivars by interspecific hybridization and back crosses with cultivated potato (Malcolmson 1969; Malcolmson and Black 1966; Müller and Black 1952). The set of eleven genes (*R1* to *R11*) was exploited in monoculture, which besides providing an efficient crop protection, was exerting a strong selection pressure on the pathogen resulting in its rapid adaptation and consequently the defeat of those *R* genes (Fry 2008; McDonald and Linde 2002);. Even, the combination of different R genes in one cultivar did not always extend the life time of the cultivar in the field. The Scottish cultivar Pentland Dell containing three R genes, *R1, R2* and *R3* was infected six years after being released (Hein et al. 2009a). There are examples of cultivars, like cv Escort, with at least 3 combined R-genes which are most of the years showing a good protection against Late Blight (Dr. R. Hutten, Wageningen UR Plant Breeding pers. comm.).

Horizontal resistance

An alternative strategy to control Late Blight is to identify and combine loci harboring genes responsible for quantitative or partial resistance traits. Quantitative resistance loci (QRL) have been largely studied and are believed to provide a more stable durable resistance (Gebhardt 1994; Leonards-Schippers et al. 1994; Oberhagemann et al. 1999); so called horizontal resistance, provided by partial resistance genes in contrast to the vertical resistance provided by major R-genes. However, Vleeshouwers et al. (2000)) showed that in backgrounds with partial resistance also HR reactions are found, suggesting the presence of R-genes with a partial effect. Late Blight QRL have been identified in many wild *Solanum* species. The Argentinean wild diploid species *S. microdontum* harbors PiQRL (Bisognin et al. 2005). *S. paucissectum* has PiQRL identified on linkage group X, XI and XII (Villamon et al. 2005). Ghislain et al (2001) mapped PiQRLs on linkage group VII and XII of *S. phureja*. Linkage group VII contains a PiQRL, identified in a population derived, from *S. demissum* (Meyer et al. 1998). Sarah Danan (2009) describes the identification in cross populations derived from *S. sparsipulim* and *S. spegazzinii*, a total of 30 Late Blight QRL present on 13 distinct genomic regions, confirming previous studies (Leonards-Schippers et al. 1992; Oberhagemann et al. 1999).

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Pyramiding of R-genes and Cisgenesis

Another way to control Late Blight disease is to learn from local farmers in the Andean region who have domesticated thousands of varieties during generations. Nowadays, over 50 varieties are cultivated on their farms using the polyculture method acting as a genetic resistance shield against Late Blight. In modern potato culture, pyramiding is pictured in mixing individual *R*-gene containing cultivars or creating a cultivar containing stacked *R*-genes.

Conventional introgression of resistance often involves inter-specific crosses and repeated backcrosses with cultivated potato, which is a very slow and inefficient process. The result is introgression of a piece of DNA with the gene of interest, surrounded by many other alleles of the donor species. This is called linkage drag and is almost always connected with linked alleles coding for traits with a negative impact (McGillivary and Clemente 1956). For durable Late Blight resistance strategies, efficient stacking of *Rpi* genes from one or several species is essential, but in practice this will enlarge the linkage drag problems considerably. Their introduction by genetic modification (GM) is a much more efficient way to improve resistance in one step and in a short period. It can even be applied to existing varieties with a long history of safe use. Currently, *Rpi* genes of natural origin, so called cisgenes (Jacobsen and Schouten 2007; Schouten and Jacobsen 2008) can be introduced using marker-free transformation systems leading to cisgenic plants with only the gene(s) of interest and without linkage drag of other genes from the wild species or from antibiotic resistance genes normally present at the T-DNA (de Vetten et al. 2003; Schaart et al. 2004; Yu et al. 2006). To date many *Rpi* genes have been cloned: *R1* and *R3a* from *S. demissum* (Ballvora et al. 2002; Huang et al. 2005a), *RB/Rpi-blb1, Rpi-blb2* and *Rpi-bt1* from *S. bulbocastanum* (Oosumi et al. 2009; Song et al. 2003; van der Vossen et al. 2003; van der Vossen et al. 2005), *Rpi-sto1* and *Rpi-pta1* from *S. stoloniferum* (Vleeshouwers et al. 2008) and *Rpi-vnt1.1, Rp-vnti1.2* and *Rpi-vnt1.3* from *S. venturii* (Foster et al. 2009; Pel et al. 2009). All belong to the NBS-LRR class of plant *R* genes, which are thought to be cytoplasmic (van der Biezen and Jones 1998). Additional *Rpi* genes that have been identified and genetically mapped, are *Rpi-blb3, Rpi-abpt, R2, R2-like* and *Rpi-mcd1* on chromosome 4 (Li et al. 1998; Park et al. 2005a; Park et al. 2005b; Park et al. 2005c; Tan et al. 2008) but also *Rpi1*from *S. pinnatissectum* on chromosome 7 (Kuhl and Hanneman 2001); *Rpi-phu1* and *Rpi-mcq1* on chromosome 9 (Sliwka et al. 2006; Smilde and Brigneti 2005) R_{ber} on chromosome 10 (Ewing and Simko 2000; Rauscher et al. 2006) and *R3b, R5, R6, R7, R8, R9, R10* and *R11* on chromosome 11 (Bradshaw et al. 2006; Huang et al. 2005b; Huang et al. 2004).

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The resistance pathway

Phytophtora infestans is an obligate biotroph which infects potato by an invaginating feeding structure (haustoria) into the host cell plasma membrane, establishing an intimate contact with this host, and delivering effector molecules (virulence effectors) into the plant cell to reprogram the plant cell in order to enhance microbial fitness. The plant reacts to this invasion by recognizing the avirulent effectors and activating the innate immunity via complex pathways that arrest proliferation of the pathogen, thus conferring disease resistance (McHale et al. 2006). The ZIGZAG model (Fig 1) in the oomycete-plant interaction (Hein et al. 2009b; Jones and Dangl 2006), nicely describes most important potato- *P-infestans* interactions. In its infection process, *P.infestans* first secretes the so-called pathogen-associated molecular patterns (PAMPs) like CBEL (the cellulose binding elicitor lectin) (Dumas et al. 2008; Gaulin et al. 2006) or elicitors like INF1 (Kamoun 2006) to trigger immunity (PTI). This process can be suppressed by secretion of effectors like the glucanase or protease inhibitor GIP2 (Damasceno et al. 2008) or EPI1 (Tian et al. 2004) which act either outside or inside host cells and re-program host metabolism to promote 'effector-triggered susceptibility' (ETS). When a successful *P. infestans* isolate has suppressed PTI, the products of plant, *Rpi* genes, as a second 'layer' of defense, can directly or indirectly detect avirulence proteins like AVR3a (Armstrong et al. 2005; Bos et al. 2006) or AVR1b (Dou et al. 2008; Shan et al. 2004). *Rpi* proteins subsequently activate resistance responses (effector-triggered immunity; ETI), often including programmed cell death during the hypersensitive response (Jones and Dangl 2006).

AVR protein detection is possible in a direct way or indirectly by the resistance genes harboring key domains for pathogen recognition, the nucleotide binding site (NBS) and the leucine rich repeat (LRR) domains (Catanzariti et al. 2010; Collier and Moffett 2009; Ellis et al. 2007; Jones and Dangl 2006; Rairdan and Moffett 2006). Although, the N terminal domain of NBS-LRR protein has been demonstrated to play a crucial role in AVR protein recognition (Moffett et al. 2009), multiple studies showed that the LRR domain is the region of the protein that confers recognition specificity (Hulbert et al. 2001; Meyers et al. 1998; Mondragon Palomino and Meyers 2002; Noel et al. 1999). In silico secondary structure of the NBS-LRR protein shows that the LRR repeats with the motif xxLxLxx form a β-strand/β-turn structure in which the x residues are exposed to solvent and are available for interactions with potential ligands (Kobe and Deisenhofer 1995). These solvent exposed residues are hyper variable and subject to diversifying selection, suggesting that these residues have evolved to detect variation in pathogen-derived ligands (Michelmore and Meyers 1998). So far five matching pairs of *PiAVR* and potato *R*-gene have been identified, *PiAVR3a/R3a* (Armstrong et al. 2005), *AVR4/ R4* (van Poppel et al. 2008), *AVR-blb1/Rpi-blb1* (Vleeshouwers et al. 2008), *AVR-blb2/ Rpi-blb2* (Oh et

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al. 2009), and *AVRvent1 /Rpi-vent1-1* (Pel 2010). Detailed investigation of the PiAVR/R interaction was so far orientated to the effector side to identify the crucial domain necessary for the activation of the hypersensitive reaction. The C terminal domains were found indispensable for PiAVR3a and AVR4 recognition (Bos et al. 2006; Van Poppel et al. 2009).

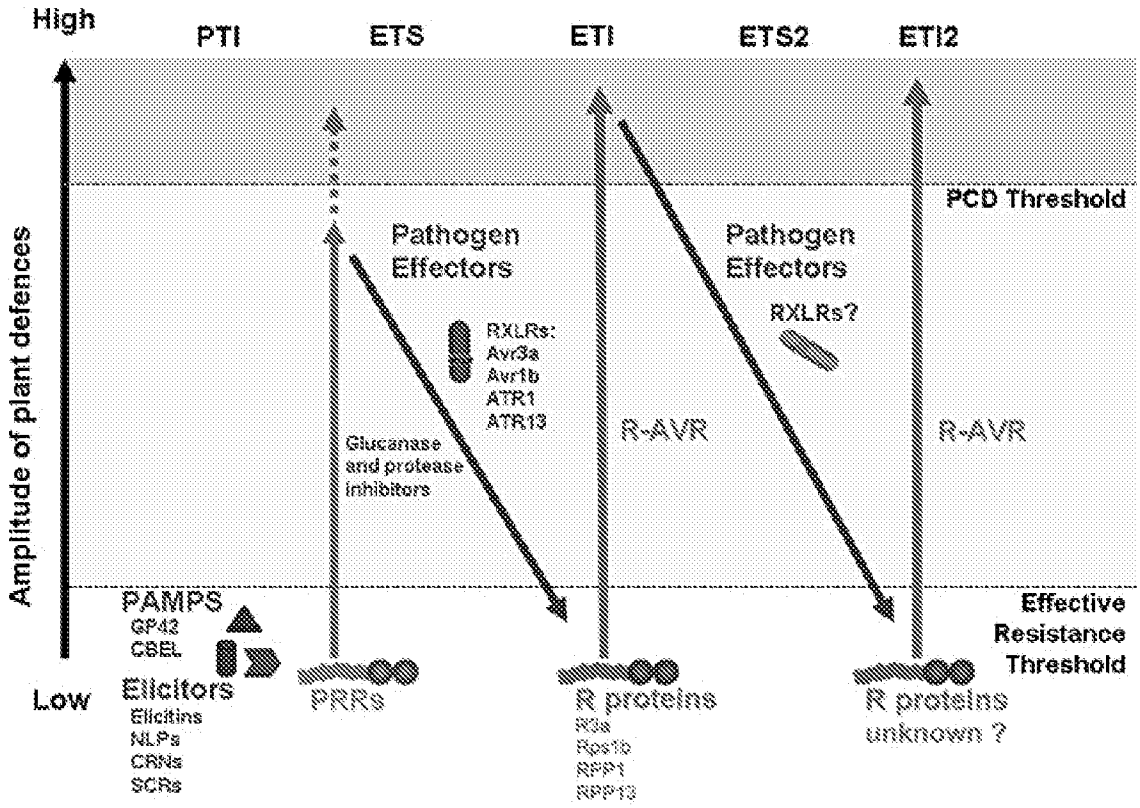


Fig. 1 The zig-zag-zig in oomycete–plant interactions. Published in Hein et al (2009b)

Outline of the thesis

The aim of the research described in this thesis was to study the major Late Blight resistance locus on linkage group IV, following the fine mapping and the characterization of the four orthologues resistance genes *Rpi-blb3, Rpi-abpt, R2* and *R2-like* (Park et al. 2005a, 2005b, 2005c; Li et al. 1998).

Cloning and functional characterization of the four genes is described in **Chapter 2,** using map based cloning and candidate gene allele mining approaches in combination with transient complementation in the heterologous *Nicotiana benthamiana* 'model' plant system. All 4 *Rpi* genes code for proteins of the NBS-LRR class of R proteins and share the highest similarity (34.9% aa) with RPP13 from *Arabidopsis thaliana* (Bittner-Eddy et al. 2000), which confers resistance to the oomycete

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H. arabidopsidis. Despite apparent differences between the four functional R proteins, all four induce a hypersensitive response upon co-expression with the *P. infestans* derived RxLR effector PiAVR2 (Gilroy et al., in preparation) in *N. benthamiana* leaves, indicating that they recognize the same effector protein

Solanum bulbocastanum is well known for its resistance to Late Blight (Niederhauser and Mills 1953), from which four Rpi-genes were so far cloned, *Rpi-blb1, Rpi-blb2, Rpi-blb3* and *Rpi-bt1* (Oosumi et al. 2009; Song et al. 2003; van der Vossen et al. 2003; van der Vossen et al. 2005). As population genetic processes and dynamics might affect the evolution of *R*-genes, a better understanding of variation within and between populations is crucial. **Chapter 3** reports about the allelic frequencies of the *Rpi-blb1*, *Rpi-blb2* and *Rpi-blb3* genes in several wild tuber-bearing *Solanum* accessions by performing allele mining with *R*-gene-specific primers. Additionally, Avr expression assays as well as detached leaf assays with key *P. infestans* isolates were utilized to strengthen the evidence for the presence of functional alleles. We related the occurrence of the genes to the geographical origin of the accessions.

Interestingly, several PiQRL mapped to the short arm of linkage group IV (Bradshaw et al. 2004; Bradshaw et al. 2006; Leonards-Schippers et al. 1994; Sandbrink et al. 2000), on the R2 locus (Hein et al. 2009a; Hein et al. 2007). Exploring the putative homology with R2GHs we attempted, in **Chapter 4**, to molecularly identify the *Rpi* genes responsible of the field resistance evaluated in a F1 population with *S. microdontum* BGRC 18302-34 (CGN 21342) as source of resistance in the earlier parentage. Molecular and phenotypic characterization of the population permitted the identification of at least three genes segregating in the population. *Rpi-mcd1.1* and *Rpi-mcd1.2* were originating from *S. microdontum* with resistance to PIC99177 and IPO-0, respectively, and *Rpi-phu2* from *S. phureja* providing only resistance to PIC99177. The use of allelic mining strategies resulted in the identification of 27 *R2* homologues, among which *Rpi-mcd1.1* was identified to be functional by using a transient complementation assay in *Nicotiana benthamiana*.

In **Chapter 5** we explored the information provided by the alleles generated in different *Rpi-blb3* allele mining strategies and used the transient co-expression system in *N. benthamiana* to identify the repeats in the LRR domain necessary for PiAVR2 recognition. Domain swaps between R genes interacting or not with PiAVR2 permitted the identification of a region spanning three LRR repeats of the cloned *Rpi* genes that determine the *R2* resistance specificity. Site directed mutagenesis pinpointed one solvent exposed amino acid to be crucial for PiAVR2 recognition.

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Chapter 2

Exploiting knowledge of R/Avr genes to rapidly clone a new LZ-NBS-LRR family of late blight resistance genes from potato linkage group IV

Anoma A Lokossou, Tae-ho Park, Gert van Arkel, Marjon Arens, Carolien Ruyter-Spira, Juan Morales, Steve C. Whisson, Paul R. J. Birch, Richard G. F. Visser, Evert Jacobsen and Edwin A. G. van der Vossen

ABSTRACT

Additionally to *Rpi-blb1* and *Rpi-blb2*, *S. bulbocastanum* appears to harbor *Rpi-blb3* located at a major late blight resistance locus on LG IV, which also harbors *Rpi-abpt, R2, R2-like* and *Rpi-mcd1* in other *Solanum* spp. Here we report the cloning and functional analyses of 4 *Rpi* genes, using a map-based cloning approach, allele mining strategy, Gateway™ technology and transient complementation assays in *N. benthamiana. Rpi-blb3, Rpi-abpt*, *R2* and *R2-like* contain all signature sequences characteristic of LZ-NBS-LRR proteins, and share amino-acid sequences 34.9% similar to RPP13 from *Arabidopsis thaliana*. The LRR domains of all 4 Rpi proteins are highly homologous whilst LZ and NBS domains are more polymorphic, those of R2 being the most divergent. Clear blocks of sequence affiliation between the four functional R proteins and those encoded by additional *Rpi-blb3* gene homologs, suggest exchange of LZ, NBS and LRR domains, underlining the modular nature of these proteins. All four Rpi genes recognize the recently identified RXLR effector PiAVR2.

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INTRODUCTION

The potato (*Solanum tuberosum)* ranks as the world's third most important food crop, after wheat and rice. Compared to these other staple crops a greater proportion of the potato crop is edible and a higher yield per hectare is achieved, which, combined with its high nutritional value, underlines its importance and explains ongoing increases in global potato production. The most significant threat to potato production worldwide is the late blight disease, caused by the oomycete pathogen *Phytophthora infestans*, which destroys leaves, stems and tubers. Current disease control relies on multiple (up to 20) fungicide applications per season (Hanson et al. 2007) and costs associated with chemical control amount to €5 billion globally per year (Whisson et al. 2001). Such pesticide inputs are both environmentally and economically undesirable and have a negative impact on the sustainability of potato production. Host resistance is a more environmentally benign means of restricting late blight infection, but the success of this management practices hinge on effective and durable host resistance and an understanding of the pathogen population.

Early in the 20th century the potential of the wild Mexican hexaploid species *Solanum demissum* was recognized for conferring extreme levels of late blight resistance, and hybrids with *S. tuberosum* were generated. Subsequently,11 major dominant resistance (*R*) genes were identified, and introgressed into potato cultivars (Müller and Black 1952; Malcolmson and Black 1966; Malcolmson 1969) However, deployment of these single genes did not provide durable resistance due to the rapidly changing populations of *P. infestans*. As an alternative approach, breeders started to select for quantitative 'field' resistance using races of *P. infestans* that were compatible with the *R* genes present in their breeding material (Toxopeus 1964; Black 1970). Despite considerable progress in genetic analysis of quantitative resistance to late blight using molecular markers (Gebhardt and Valkonen 2001; Simko 2002) under long day conditions breeders have achieved little progress, the major draw backs being tetrasomic inheritance of potato and the strong linkage between foliage resistance and late foliage maturity (Oberhagemann et al. 1999; Visker et al. 2003; Bradshaw et al. 2004). It is therefore anticipated that breeding for late blight resistance in potato, aimed at substantially contributing to disease management, requires the deployment of major resistance to *P. infestans* (*Rpi*) genes. Recent germplasm screens are revealing a wealth of *Rpi* genes in wild *Solanum* species and the challenge now is how to select, judiciously combine, and deploy sets of *Rpi* genes that can confer durable late blight resistance in modern potato varieties.

Key to durable exploitation of *Rpi* genes is also a better understanding of their interactions with effectors from *P. infestans.* All microbes trigger immune responses in plants *via* host receptor-mediated recognition of pathogen-associated molecular patterns (PAMPs). Such PAMP-triggered

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immunity (PTI) can be suppressed by secretion of effectors which act either outside or inside host cells and re-program host metabolism to promote 'effector-triggered susceptibility' (ETS). When a successful pathogen has suppressed PTI, the products of plant, *R* genes, as a second 'layer' of defense, can directly or indirectly detect effectors (termed avirulence proteins; AVRs). R proteins subsequently activate resistance responses (effector-triggered immunity; ETI), often including programmed cell death during the hypersensitive response (Jones and Dangl 2006). The ability of a pathogen to evade recognition in the *R*-gene-for-*Avr*-gene concept (Flor 1971), can be explained by an evolutionary 'arms race' (Bergelson et al. 2001; Holub 2001). The generation, through mutation, and selection of *AVR* alleles that evade recognition is matched by strong selection pressure for *R* gene alleles that retain recognition. This is particularly evident in the case ATR1-RPP1 and ATR13-RPP13 interaction in the *Hyaloperonospora arabidopsidis* (formerly *H. parasitica*)-Arabidopsis pathosystem, where considerable allelic diversity has been revealed by studying natural host and pathogen populations (Rehmany et al. 2005; Allen et al.2008). In naturally co-evolving *P. infestans*-potato populations, similar high levels of allelic diversity are expected to be observed. So far, studies of the PiAVR3a-R3a interaction reveal diversifying selection for both genes (Amstrong et al. 2005; Huang et al, 2005). Recently, several oomycete *AVR* genes were identified (Allen et al. 2004; Shan et al. 2004; Armstrong et al. 2005; Rehmany et al. 2005; Vleeshouwers et al. 2008). Although each of the AVR proteins is distinct at the level of primary sequence similarity, they all share a secretion signal peptide followed by the motif RXLR and an acidic region, often ending in the sequence EER (Whisson et al. 2007; Birch et al. 2008a; Birch et al. 2008b).The RXLR-EER region is required for translocation of PiAVR3a, following its secretion from *P. infestans* haustoria, to the inside of host plant cells (Whisson et al. 2007). Translocation of RXLR-containing proteins inside the plant cell is consistent with their recognition by host NBS-LRR resistance proteins (Birch et al. 2006). As all oomycete AVR proteins identified to date have the RXLR motif, it provides a signature to identify AVR candidates from the *P. infestans* genome. Future knowledge of RXLR effectors diversity and their survey in *Phytophtora infestans* population could predict the span life of Rpi-genes in specific areas.

Conventional introgression of resistance often involves inter-specific crosses and repeated backcrosses with cultivated potato, which is a very slow and inefficient process. Currently, *Rpi* genes of natural origin, so called cisgenes (Jacobsen and Schouten 2007; Schouten and Jacobsen 2008), can be introduced using marker-free transformation systems leading to cisgenic plants with only the gene(s) of interest and without linkage drag of other genes from the wild species or from antibiotic resistance genes normally present at the T-DNA (de Vetten et al. 2003; Schaart et al. 2004; Yu et al. 2006). To date six *Rpi* genes have been reported: *R1* and *R3a* from *S. demissum* (Ballvora et al. 2002; Huang et al. 2005) *RB/Rpi-blb1* and *Rpi-blb2* from *S. bulbocastanum* (Song et al. 2003; van der Vossen

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et al. 2003; van der Vossen et al. 2005),and *Rpi-sto1* and *Rpi-pta1* from *S. stoloniferum* and *S. papita,* respectively (Vleeshouwers et al. 2008). All belong to the NBS-LRR class of plant R proteins, which are thought to be cytoplasmic (van der Biezen and Jones 1998). Various loci are reported to contain *Rpi* genes on linkage group (LG) VII (Kuhl and Hanneman 2001), LG IX (Smilde and Brigneti 2005; Sliwka et al. 2006), LG X (Ewing and Simko 2000; Rauscher et al. 2006) and LG XI (Huang et al. 2004; Huang 2005; Bradshaw et al. 2006).

Here we describe the cloning and functional characterization of *Rpi-blb3, Rpi-abpt, R2* and *R2-like* from the major late blight resistance locus on LG IV of potato (Park et al. 2005a, 2005b, 2005c; Li et al. 1998). Upon map based cloning of *Rpi-blb3*, an efficient candidate gene allele mining approach in combination with transient complementation in the heterologous *Nicotiana benthamiana* 'model' system lead to the rapid cloning of the other three genes. All 4 *Rpi* genes code for proteins of the NBS-LRR class of R proteins. Interestingly, with respect to known functional R proteins, they share the highest similarity (34.9% aa) with RPP13 from *Arabidopsis thaliana* (Bittner-Eddy et al. 2000), which confers resistance to the oomycete *H. arabidopsidis.* Despite apparent differences between the four functional R proteins, all four induce a hypersensitive response upon co-expression with the *P. infestans* derived RxLR effector PiAVR2 (personal communication, E. Gilroy) in *N. benthamiana* leaves, indicating that they recognize the same effector protein.

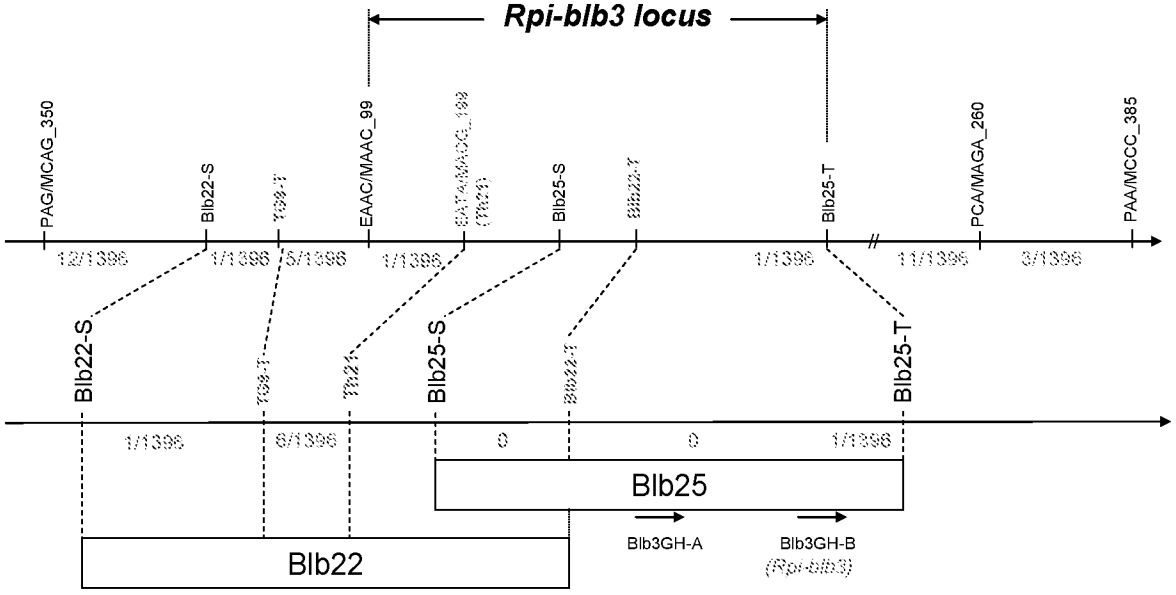
RESULTS

Cloning of Rpi-blb3 and Rpi-abpt

In order to clone *Rpi-blb3* and *Rpi-abpt*, two BAC libraries were constructed using DNA derived from the resistant clones Blb99-256-3 and 707TG11-1, respectively. Approximately 74000 clones with an average insert size of 85 kb, corresponding to 8 genome equivalents, were obtained for each library. These libraries were screened initially with SCAR marker Th21, which co-segregated with resistance in mapping populations of 1396 and 1383 F1 progeny plants, respectively (Park et al. 2005a). In this way BAC clones Blb22 and TG9 were identified, respectively (Fig. 1A and 1B). By sequencing the ends of these two BACs, new markers were developed which were used to define the genetic intervals of the R loci more precisely and to re-screen the BAC libraries to identify clones that overlapped with the initial BAC clones. In this way the *Rpi-blb3* locus was delimited to a 0.1 cM interval (EAAC/MAAC_99- Blb25-T; 2/1396 recombinants) that is physically spanned by the two partially overlapping BAC clones Blb22 and Blb25 (Fig. 1A). In the case of the *Rpi-abpt* locus, the partially

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A.



B.

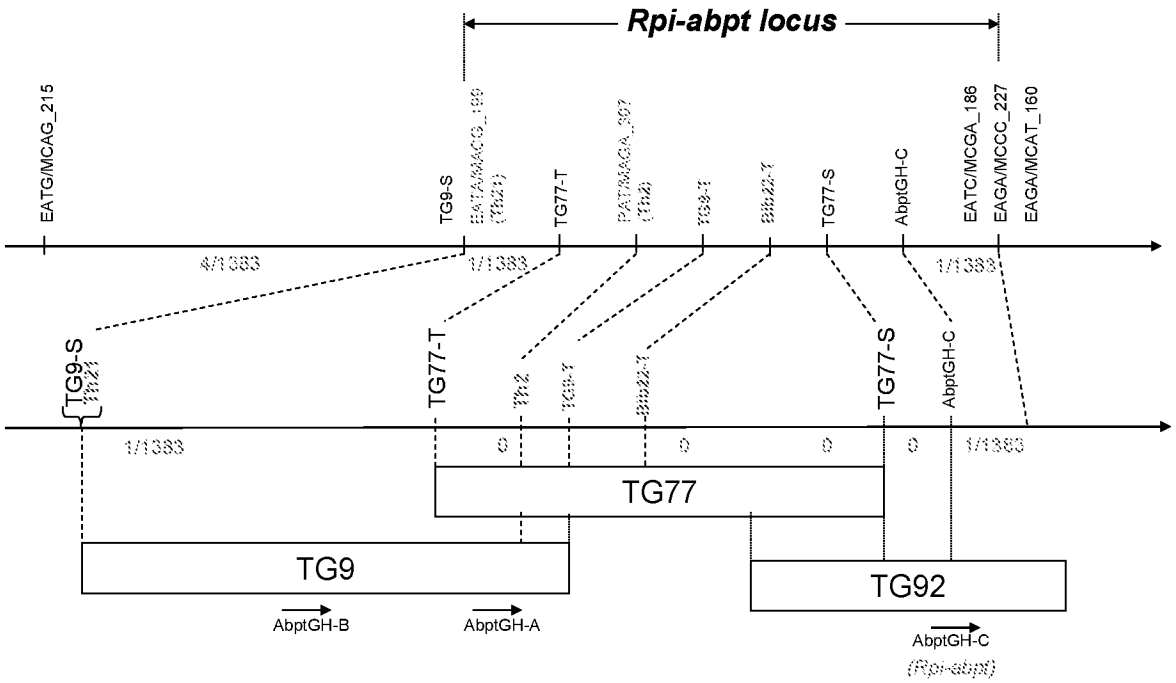


Figure 1: Genetic and physical map of the *Rpi-blb3* (A) and the *Rpi-abpt* (B) loci. Indicated are the relative positions of markers (in green are the one used in the mapping of both genes), the number of recombinants identified between markers, the overlapping BAC clones defining the two loci, and the relative positions of the *R* gene candidates that have been targeted for complementation analysis

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overlapping BAC clones TG9 and TG77 were identified. One end of the contig co-segregated with resistance (TG77-S) whilst the other mapped 0.1 cM proximal to *Rpi-abpt* (TG9-S) (Fig. 1B).

Table 1. Overview of markers and primers used for mapping and cloning of *Rpi-blb3*

Name	Type	Polymerase chain reaction primer (5′ to 3′)	Tm[a]	Enzyme[b]
Th21	SCAR	F: ATTCAAAATTCTAGTTCCGCC		a.s.
		R: AACGGCAAAAAAGCACCAC		
Blb22-S	CAPS	F: GTTTGATGTATGTTTGTTCTTGC	56	Msp1
		R: TAATGCACTAATAACTAACTAGG		
Blb22-T	SCAR	F: CTTTATTAGTTCCAAGAGCTAC	56	
		R: ACCCATCCCTTTTTCCTTATC		
Blb25-S	CAPS	F: ACAGATGCTACGTCCATCAC	56	Alu1
		R: CTCCACATGCGATGCAAAAAG		
Blb25-T	CAPS	F: TTTCGATTATGGTGAGCCTTC	56	Hpy 188
		R: TAGAAAAAGGGTGGTTGTGAC		
RGH1	CAPS	F: GGSAAGACCACTCTTGCAAG	50	HpyCH4IV
		R: GGTTTTTAAGCTGCTAATGTTG		
RGH2	SCAR	F: GGSAAGACCACTCTTGCAAG	50	a.s.
		R: TGGTYATAATYACTCTGCTGC		
RGH3	CAPS	F: ATGRCTGATGCMTTTRTGTC	50	HaeIII
		R: CCYAAGTASAGAAAACACTGC		
4-PLOOP		F: GGiATGGGiGGiYTiGGiARGAC	68	
4-GLPL		R: TACiACAATiGCAAGiGGTAAMCC		
4-GLO2		F: GTGTCTCTCAAGAGTACAACAC	56	
		R: GCTCGAACATCAAGTAGTTTCC		
Blb3-start		F: ATGGCTGATGCCTTTCTRTCATTTG	55	
Blb3-end		R: TCAGGAATCTCCTTTAAATTTGGAC		
Blb3-prom		F: TCTTCCTTAGCATTCGTAGC	55	
		R: CTTTAGGAATACTAGTTTTGATTG		
Blb3-ter		F: AGCTTTTCTGCCAAGCACATTGG	55	
		R: GTACCCTCCGTTTGTCGTTTGATC		
Blb3-LRR-1-8		F: CTCTTTATGTATCAGACATGGC	55	
		R: CAACATCTTTCCACTGATCAC		
Blb3-prom -end		F: CCCCAAGTTGTATAATGGTTG	55	
Blb3-orf-bg		R: TGCTTGAGTGATTGAATCTCC		
Sto-orf-bg		R: GGCCATATTCAGACTGGGAG		
Blb3-spe		F: AGCTTTTTGAGTGTGTAATTGG	55	
		R: GTAACTACGGACTCGAGGG		

[a] Annealing temperature. In AF411807R, annealing temperature was 48°C for the first 7 cycles and 54°C for the last 30 cycles.

[b] Restriction enzymes that reveal polymorphism between resistant and susceptible linked alleles of the marker. a.s: allele-specific marker showing polymorphism without digestion

To gain insight into the molecular structure of the *R* loci under study, BAC clones Blb22 and TG9 were sequenced to 6x coverage. This revealed that clone Blb22 did not contain any *R* gene

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homologues (RGH) whereas clone TG9 contained two RGH, which shared significant similarity to RGHs present in the sequenced tomato BAC clone AF411807L (Van der Hoeven et al. 2002) (Fig. 1). BAC clones Blb25 and TG77 were subsequently screened for the presence of RGH-specific sequences through PCR analysis using the primers 4-PLOOP-F and 4-GLPL-R (Table 1) which were designed by aligning the RGH sequences of clone AF411807L with those present on BAC clone TG9. Southern analysis of BAC clones Blb22, Blb25, TG9 and TG77 using an RGH-specific PCR fragment amplified from BAC clone Blb25 as a probe identified a minimum of two RGHs on BAC Blb25 and TG9 and one RGH on TG77 located on the overlapping part with TG9 (Fig. 1).Libraries harboring random overlapping binary subclones of 8-10 kb were therefore generated from BAC clones Blb25 and TG9. A total of 1152 clones per library were screened for the presence of RGHs using primers 4-GLO2-F and 4-GLO2-R (Table 1). Based on restriction analyses of the PCR fragments, RGH positive subclones were divided into separate classes: *Blb3GH-A* and *Blb3GH-B* for *Rpi-blb3*, *AbptGH-A* and *AbptGH-B* for *Rpi-abpt*. After determining the relative positions of the RGHs within the 8-13 kb subclones, candidates from each class were targeted for transformation to the susceptible potato cultivar Desiree. Transformation experiments carried out with subclones harboring candidates *Blb3GH-A*, *Blb3GH-B*, *AbptGH-A* and *AbptGH-B*, respectively, resulted in numerous primary transformants. Detached leaf assays using isolates IPO-0 and 90128 revealed that all of the plants transformed with subclones harboring candidates *Blb3GH-A*, *AbptGH-A* and *AbptGH-B* were susceptible to both isolates but that the majority (7/8) of the tested transgenic plants harboring *Blb3GH-B* reacted to both isolates with a hypersensitive response (HR) (Fig. 2). In view of the differential response between the primary transformants harboring *Blb3GH-A* and *Blb3GH-B*, we designated *Blb3GH-B* as the *Rpi-blb3* gene.

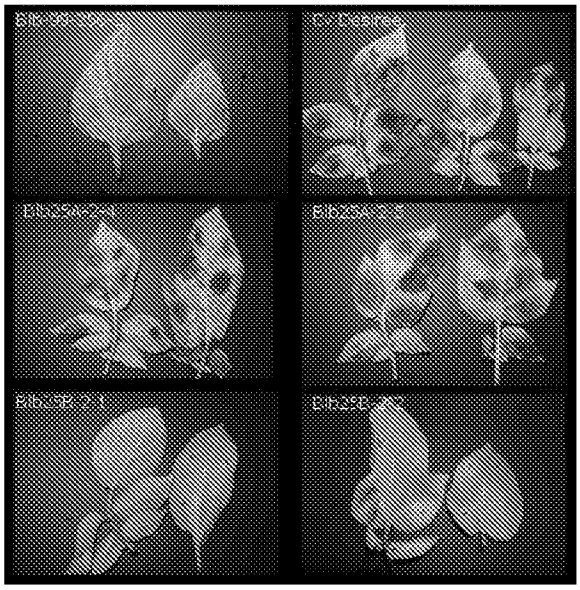


Figure 2: Genetic complementation for late blight resistance. Eight days after inoculation with *Phytophtora infestans* isolate 90128, large necrotic lesions and massive sporulation were noted for potato cultivar Desiree, and derived transformant Blb25A-2-4 and Blb25A-2-5, both expressing RGH-Blb25A. Typical resistance phenotype, i.e hypersensitive response (HR) was observed for Blb25B-2-1 and Blb25B-2-2 expressing RGH-Blb25B (*Rpi-blb3*)

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In order to identify additional candidate genes for *Rpi-abpt*, the *Rpi-abpt*-specific BAC library was screened with TG77-S, leading to the identification of the TG77 overlapping BAC clone TG92 (Fig. 1B). Screening of this BAC clone with different sets of primers designed to amplify *AbptGH-A, AbptGH-B, Blb3GH-A* or *Rpi-blb3*, resulted in the identification of a third *Rpi-abpt* candidate gene, *AbptGH-C* (Fig. 1B). *AbptGH-C* LRR domain was amplified, cloned and sequenced using the primers Blb3-LRR-1-8 (Table 1). Sequence comparison of the three *abpt-GHs* enabled to design *AbptGH-C* specific marker (Fig. 1B) which co-segregated with resistance. Southern blot analysis using the AbptGH-C amplicon as a probe revealed that clone TG92 contained one resistance gene encompassing an LRR domain highly similar to the one of *AbptGH-C*. Primers designed on the start and stop codon of *Rpi-blb3* were subsequently used to amplify a full-length AbptGH-C amplicon from clone TG92, which was cloned into the Gateway® entry vector pDONR221. Using Multisite Gateway® technology, the *AbptGH-C* amplicon was subsequently cloned into the binary pKGW-MG destination vector between *Rpi-blb3* derived promoter and terminator sequences of 2723 nt and 883 nt, which were cloned into pDONR™ P4-P1R and pDONR™ P2R-P3 (Suppl. Fig. S1).

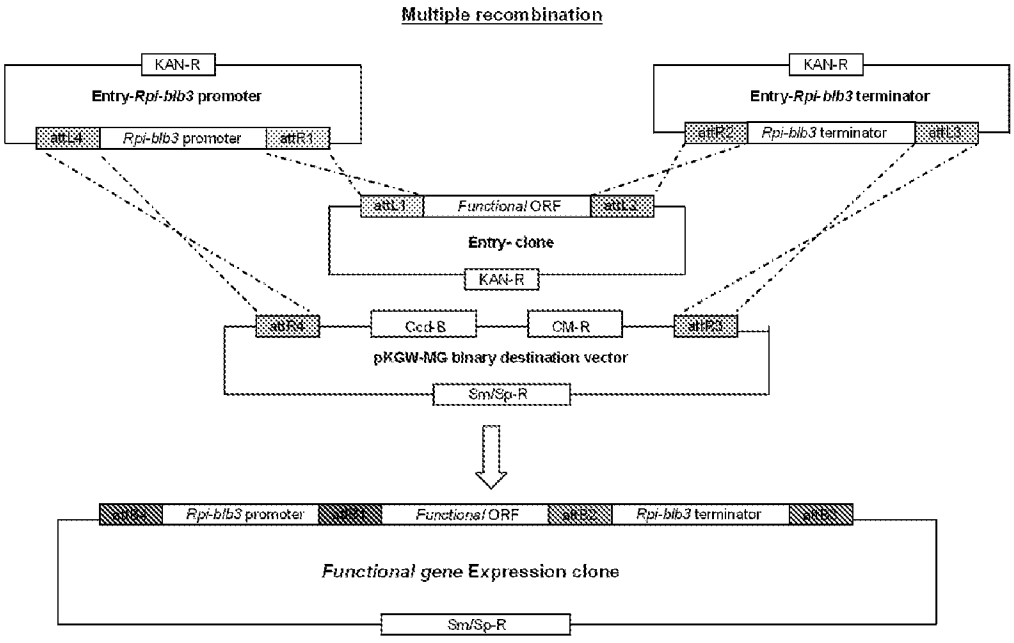


Figure S1: Gateway strategy used to clone *Rpi-abpt*, *R2* and *R2-like*. LR recombination of the three entry clones bearing the DNA fragments *Rpi-blb3* promoter, *Functional* ORF and *Rpi-blb3* terminator with the Multisite Gateway destination binary vector pKGW-MG, leading to the functional gene expression clone.

Complementation analysis was carried out in *N. benthamiana* using the *Agrobacterium tumefaciens* transient assay (ATTA) whereby leaves from 4-week-old wild type *N. benthamiana* plants were infiltrated with the *Agrobacterium* strain COR308 containing pKGW-AbptGH-C. The binary

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clones pBP-Rpi-blb3 and pKGW-Rpi-blb3, comprising the genomic *Rpi-blb3* gene and a Multisite Gateway® reconstituted *Rpi-blb3* gene construct, respectively, were used as positive controls, and pBP-AbptGH-A as a negative control. After two days, infiltrated leaves were inoculated with *P. infestans* strain PY23, IPO-0 and IPO-C in detached leaf assays. Leaves infiltrated with pKGW-AbptGH-C, pBP-Rpi-blb3 and pKGW-Rpi-blb3 developed HRs at the inoculation sites whereas non infiltrated leaves and those infiltrated with pBP-AbptGH-A were susceptible to isolate PY23 and IPO-0. As expected, all leaves inoculated with IPO-C were susceptible (Fig. 3 and Table 2). In view of these results, *AbptGH-C* was designated *Rpi-abpt*.

Allele mining for R2 and R2-like

Rpi-blb3 and *Rpi-abpt* belong to the major late blight resistance locus on LG IV that also harbors *R2* and *R2-like* (Li et al. 1998; Park et al. 2005c). In view of the conserved marker order and observed allelic conservation between the genetic maps of *Rpi-blb3, Rpi-abpt*, *R2*, and *R2-like* (Park et al. 2005c), and the high sequence conservation between *Rpi-blb3* and *Rpi-abpt*, we hypothesised that *R2* and *R2-like* were orthologues of *Rpi-blb3* and thus attempted to clone them through an allele mining strategy. The same primers used to amplify the *Rpi-abpt* candidate gene from BAC clone TG92 were used to amplify full-length Blb3GH from the resistant parental genotypes CEBECO44158-4 (MaR2) and AM3778-16, harboring *R2* and *R2-like*, respectively. Amplicons of the expected size were cloned into pDONR221 and fully sequenced. In total, eight unique sequences were obtained from AM3778-16 (R2-likeGH) and nineteen from CEBECO44158-4 (R2GH), with amino acid identities between Rpi-blb3 and the novel Blb3GH ranging from 86.4% to 97.3% for R2-likeGH and 83.8% to 94.2% for R2GH (Supplementary Table S1 and S2). Phylogenetic analysis of all the available amino acid sequences clustered one R2-like-GH and five R2GH in a clade together with the functional proteins Rpi-blb3 and Rpi-abpt (Fig. 4). The amino acid sequence of R2-likeGH-8 shares 97.3 % amino acid identity with Rpi-blb3. R2GH-2, R2GH-8, R2GH-G3, R2GH-D3 and R2GH-65 share 94.2, 91, 92.6, 89.7, and 92.8% amino acid identity with Rpi-blb3, respectively. This set of candidate genes was targeted for functional analysis and therefore cloned into the binary vector pKGW-MG between the *Rpi-blb3* promoter and terminator sequences, as described above for the *Rpi-abpt* gene.

Transient complementation assays in *N. benthamiana* indicated that R2GH-G3 and R2-likeGH-8 conferred resistance to Pi isolate IPO-0, whereas R2GH-2, R2GH-8, R2GH-D3 and R2GH-65 were non-functional (Fig. 3). R2GH-G3 and R2-likeGH-8 were therefore designated *R2* and *R2-like*, respective.

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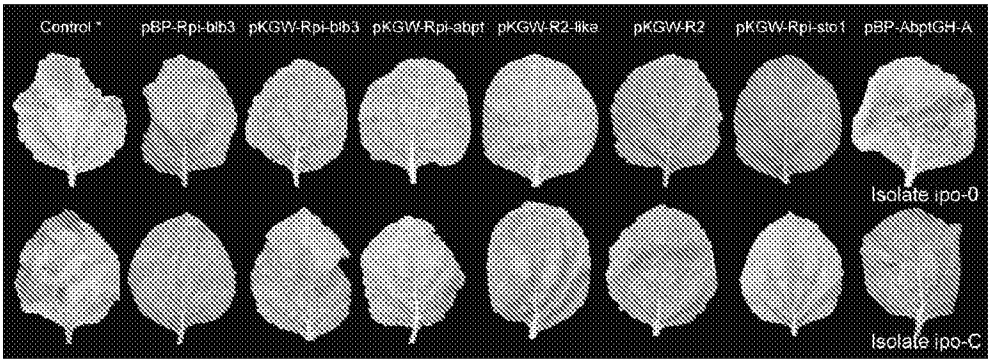


Figure 3. Complementation of *Rpi-blb3, Rpi-abpt , R2, R2-like, Rpi-sto1* by transient expression in *Nicotiana benthamiana*, followed by inoculation by *Phytophtora infestans* isolate Ipo0 (avirulent) and Ipo-C (virulent on all except *Rpi-sto1*). The prefix pBP stand for pBINplus, and pKGW stand for the gateway destination vector containing *Rpi-blb3* regulatory elements. In leaves expressing pB-Rpi-blb3, pKGW-Rpi-blb3, pKGW-Rpi-abpt, pKGW-R2-like, pKGW-R2, a HR was observed at seven days post inoculation with Pi isolate Ipo-0, whereas large necrotic lesions and massive sporulation were evident for Pi isolate Ipo-C. Wild type *N. benthamiana* (Control *) and the non functional AbptGH-A (pBP-AbptGH-A) were included as susceptible controls, and Rpi-sto1 (pKGW-Rpi-sto1) was included as a resistant control **.**

Table 2 Overview of resistance screening with a set of 16 *P. infestans* isolates[a].

spp	Genotype	Gene	Chr.	py 23	IPO-0	90128	H30P04	VK98014	IPO428-2	Dintel	Katshaar	F95573	EC1	89148-09	99183	99189[b]	IPO-C	USA618	99177
BLB	Blb99-256-3	*Rpi-blb3*	4	R	R	R	R	R	R	R	R	R	R	R	R	S	S	S	S
BLB	707TG11-1	*Rpi-abpt*	4	R	R	R	R	R	R	R	R	R	R	R	R	**R**	S	S	S
BLB	AM3778-16	*R2-like*	4	R	R	R	R	R	R	R	R	R	R	R	R	S	S	S	S
DMS	CEBECO44158-4	*R2*	4	R	R	R	R	Rq	R	Rq	R	R	R	R	R	S	S	S	S
	cv. Desiree	-	-	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
	cv. Bintje	-	-	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S

[a] Differential specificity are in bold. Chr.=chromosome, S= susceptible; R= resistant and Rq= quantitatively resistant.

[b] Isolate differentially recognized

All four R genes interact with the RXLR-EER effector PiAVR2

Using a map-based cloning approach, markers flanking the *PiAVR2* locus (van der Lee et al., 1997) were used to delimit a physical region of 742,635 bp of the *P. infestans* genome sequence spanning the genetic interval containing *PiAVR2*. Within this region, two candidate *PiAVR2* genes on supercontig 16 of the *P. infestans* genome (16_7987 and 16_8044), each encoding a secreted RXLR-EER effector, were identified. The cloning and analyses of these gene candidates will be reported in detail in a separate publication (personal communication, E. Gilroy).

To functionally analyze the two RxLR-EER candidate genes identified at the *PiAVR2* locus in this study, *N. benthamiana* leaves were co-infiltrated with *A. tumefaciens* strains carrying a binary construct expressing one of the four *Rpi* genes under study together with strains carrying a binary construct expressing each of the two candidate RxLR-EER genes. Co-infiltration of *Rpi-blb3, Rpi-abpt, R2* and *R2-like* with *16_7987* resulted in a hypersensitive cell death response (Fig. 5), whereas co-expression of these *Rpi* genes with the *16_8044* candidate or *PiAVR3a*, failed to elicit visible cell death (Fig. 5; personal communication, E. Gilroy). Based on these results *16_7987* was designated *PiAVR2*, and clearly all four *Rpi* genes under study interact with *PiAVR2*.

Gene structure and functionality

Rpi-blb3, Rpi-abpt, *R2* and *R2-like* are intronless genes which encode ORFs of 2538-2544 nucleotides (nt) that code for proteins of 845-847 amino acids harboring all the signature sequences characteristic of LZ-NBS-LRR R-proteins (Fig. 6). Interestingly, with respect to known functional R proteins, Rpi-blb3, Rpi-abpt, R2, and R2-like share the highest similarity (34.9% aa) with RPP13 from *Arabidopsis thaliana* (Bittner-Eddy et al. 2000), which confers resistance to *H. arabidopsidis* isolates containing *ATR13*. The highest similarity with RPP13 resides in the NBS domain with 49.3% sequence identity, and the lowest within the LRR domain (34.3%). The LRR domains of Rpi-blb3, Rpi-abpt, R2, and R2-like are highly homologous and comprise 14 imperfect repeats (Fig. 6). The LZ and NBS domains are more polymorphic, those of R2 being the most divergent. Rpi-abpt and R2-like are identical except for the sequence between LRR2 and LRR3, where Rpi-abpt contains a stretch of amino acids that is identical to that of R2. The LRR domain of R2 is identical to that of Rpi-abpt (Fig. 6).

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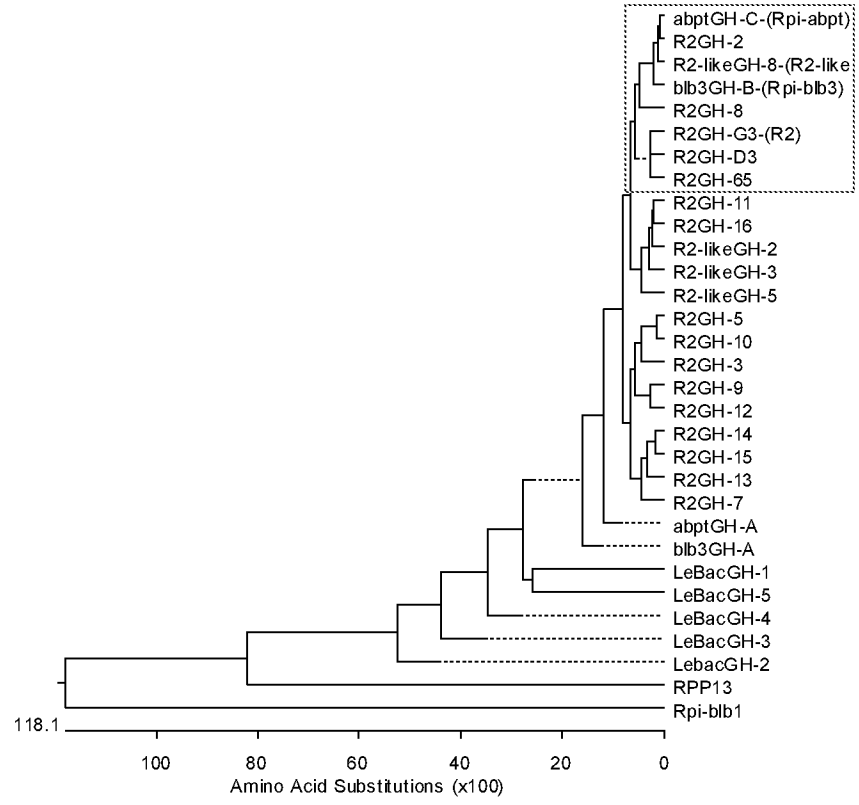


Figure 4: Phylogenetic tree at the amino acid level of Rpi-blb3, RGHs amplified from late blight resistant potato clones harboring *Rpi-abpt*, *R2-like or R2*, RGHs present on the tomato Bac clone AF411807, *RPP13-Nd* and *Rpi-blb1*. Boxed is the group containing Rpi-blb3, Rpi-abpt, R2 and R2-like.

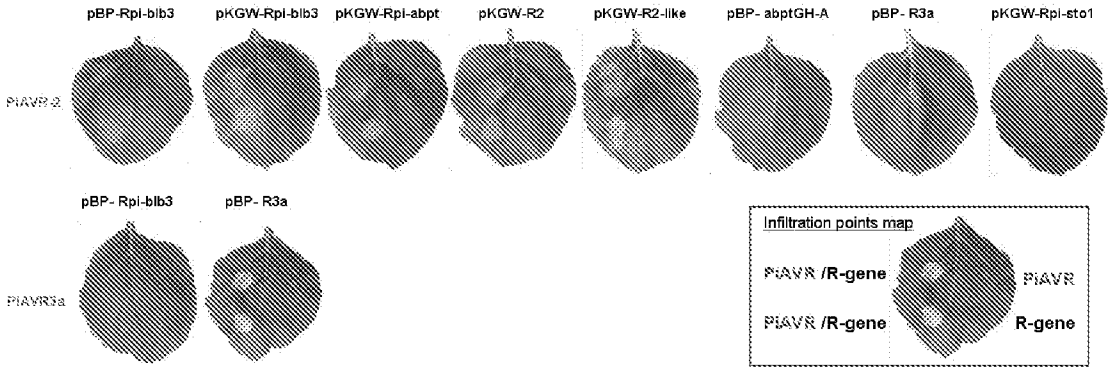
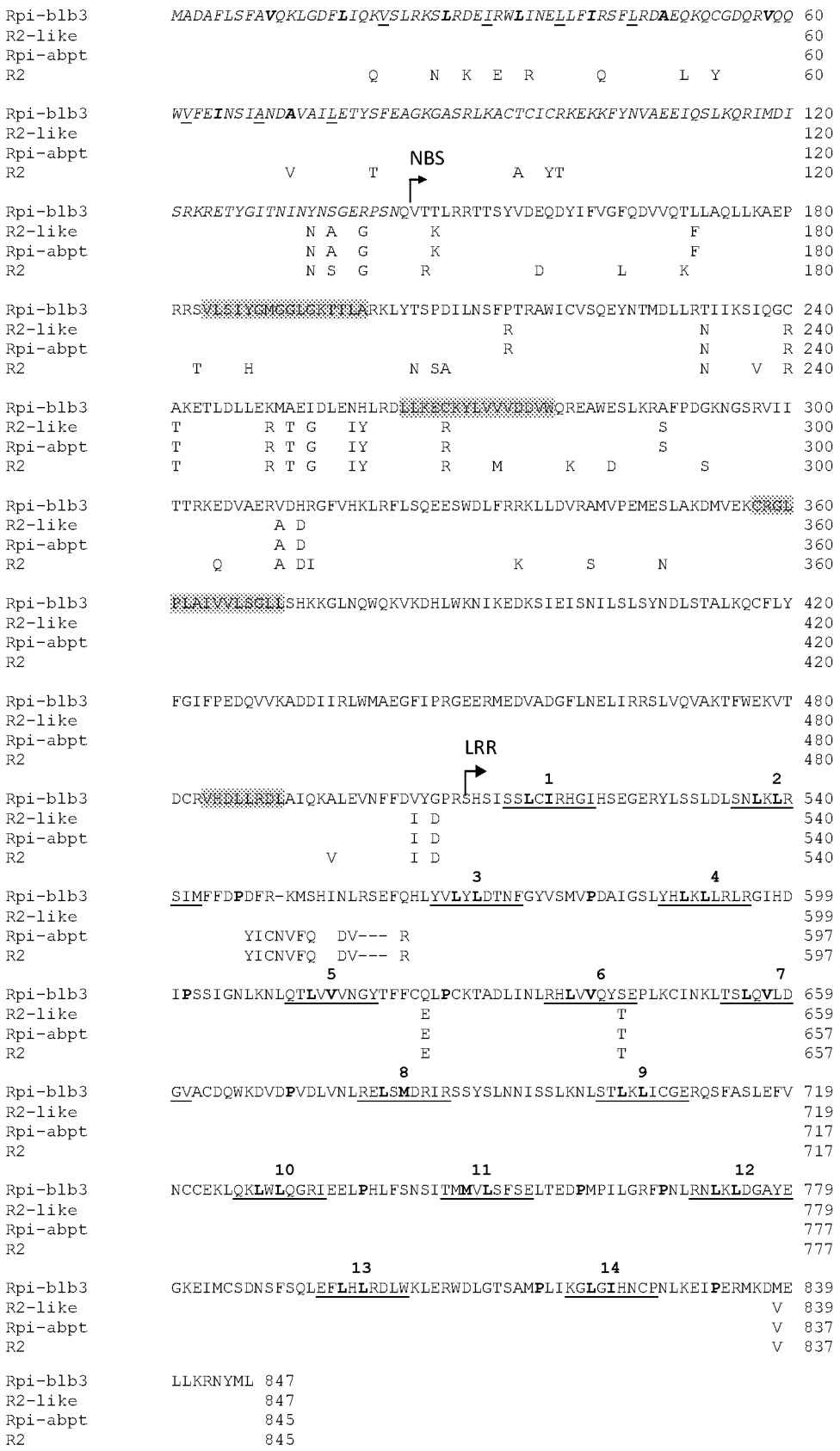


Figure 5. Agrobacterium-mediated co-expression of *PiAvr2* or *PiAvr3a* together with *Rpi-blb3, Rpi-abpt , R2, R2-like, Rpi-sto1* , the non functional *abptGH-A* or *R3a*. The different infiltration points are indicated on the map. On each leaf, four infiltration spots were performed, duplicated co-infiltration of the *Avr* and the *R* gene on the left , the *Avr* on the top right and the *R* gene on the bottom right. *Rpi-blb3, Rpi-abpt , R2 and R2-like* in combination with *PiAvr2* as well as *R3a* and *PiAvr3a* induced the hypersensitive response (HR). Pictures were taken 6 days post-infiltration.

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Figure 6: Alignment of the deduced protein product encoded by the functional genes *Rpi-blb3*, *Rpi-abpt*, *R2-like*, and *R2*. The full amino acid sequence of *Rpi-blb3* and in comparison, differing amino acids of *Rpi-abpt, R2-like*, and *R2* are shown. In italics is the leucine zipper domain with the first and the fifth hydrophobic residues from the heptads in bold and underlined respectively. Positions of the nucleotide binding (NB)-ARC and the leucine reach repeat (LRR) are indicated by arrows. The NB-ARC domain has its characteristic conserved domains highlighted in grey (kinase 1a, kinase 2 and kinase 3). The LRR domain domain is composed of 14 repeats with the motif xx**L**x**L**xxx .

Alignment of the nucleotide sequences of the four functional genes and those of four additional *Blb3GHs* and subsequent analysis of informative polymorphic sites (IPS), i.e. sites where two or more genes carry the same nucleotide (Parniske et al. 1997), reveals clear blocks of sequence affiliation between different members of the gene family (Fig. S2), indicating that sequence exchange events between *Blb3GHs* have been involved in the evolution of the gene family (Fig. S2). Interestingly, the observed sequence affiliations in the 5'-terminal half of the genes extend throughout the LZ and NBS domains whereas sequence affiliations in the LRR domain suggest exchange of specific combinations of LRRs, underlining the modular nature of R proteins.

In an attempt to assess the biological relevance of the observed sequence exchange events in relation to resistance spectrum, detached leaves of the parental clones harboring *Rpi-blb3* (Blb99-256-3), *Rpi-abpt* (707TG11), *R2* (CEBECO44158-4(MaR2)) and *R2-like* (AM3778-16) were inoculated with 16 different *P. infestans* isolates (Table 2). For 15 isolates all four clones displayed the same specificity. However, one isolate, PIC99189, displayed a differential interaction; Blb99-256-3, AM3778-16 and CEBECO44158-4(MaR2) were susceptible to this isolate whereas 707TG11-1 was resistant (Table 2). Previously it was reported that 707TG11-1 contained tuber specific *R1-like* (Park et al. 2005) and foliage specific *R3-haplotype* (Huang . 2005). To dissect the resistance specificity of 707TG11-1 we inoculated 48 F1 progeny plants of the *Rpi-abpt* mapping population RH4x103 with isolates 90128 and PIC99189. For both isolates a clear 1:1 segregation of resistance was observed but there was no correlation between resistance to both isolates, confirming that the resistance to isolate PIC99189 is indeed the result of a second gene other than *Rpi-abpt*, and not located on LG IV.

In order to confirm the functionality of *Rpi-abpt*, *R2-like*, and *R2*, transgenic Desiree were generated. Detached leaves of 6, 5, and 2 independent transgenic lines expressing *R2-like, Rpi-abpt*, and *R2,* respectively, were inoculated with *P. infestans* isolates IPO-0 and IPO-C. All transformants reacted with a hypersensitive response (HR)to isolate IPO-0 and were susceptible to isolate IPO-C (Fig S3). This result confirms that *Rpi-abpt*, *R2-like*, and *R2* are functional *R* genes with expected specificity to *P. infestans* isolates. Moreover, the use of the *N. benthamiana* transient complementation assay as an efficient tool for rapid functional screen of putative R-genes is verified."

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Supplementary Table S2: Amino acid sequence similarity between the LZ, NBS or LRR domains of the different homologues depicted in the phylogenetic tree (Fig. 4) (Rpi-abpt, R2-like, R2, blb3GH-A, abptGH-A, 3 R2likeGHs, 15 R2GHs, 5 LeBacGHs, RPP13, Rpi-blb1) and Rpi-blb3. Highlighted in grey are the genes depicted in the crustal W alignment (Fig. 6).

blb3GH-B -(Rpi-blb3)	blb3GH-B-(Rpi-blb3)	abptGH-C -(Rpi-abpt)	R2-likeGH-1 -(R2-like)	R2GH-G3 -(R2)	blb3GH-A	abptGH-A	R2-likeGH-2	R2-likeGH-3	R2-likeGH-5	R2GH-2	R2GH-3	R2GH-5	R2GH-7	R2GH-8	R2GH-9	R2GH-10
LZ-NBS-LRR	100	95.9	97.3	92.6	81.7	86.3	86.4	89.9	91.4	94.2	84.7	87.9	88.2	91	86.6	86.8
LZ	100	98	98	90.1	87.5	90.8	84.2	84.2	84.2	98	90.1	90.1	97.4	89.5	90.1	89.5
NBS	100	95.5	95.5	91.2	91.8	93.6	89.9	89.7	91.8	94.7	89.4	93.4	92.6	91	89.7	93.4
LRR	100	95.9	99	95.9	81.9	80.4	85.3	92.8	94	93	81.4	83.8	82.6	93.7	84.5	81.9

blb3GH-B-(Rpi-blb3)	blb3GH-B-(Rpi-blb3)	R2GH-11	R2GH-12	R2GH-13	R2GH-14	R2GH-15	R2GH-16	R2GH-D3	R2GH-65	LeBacGH-1	LebacGH-2	LeBacGH-3	LeBacGH-4	LeBacGH-5	RPP13	Rpi-blb1
LZ-NBS-LRR	100	87	85	86.9	84	83.8	86.7	89.7	92.8	75.6	55.7	70.9	78.3	47.6	34.9	24.4
LZ	100	90.8	90.1	98	88.8	90.8	85.5	89.5	90.1	80.9	79.6	76.3	78.3	50	34.9	25
NBS	100	89.4	89.1	88.9	89.1	88.1	89.4	90.2	92.3	80.6	59.7	81.7	86.7	54.9	49.3	31.8
LRR	100	85.7	81.9	83.1	80.4	80.4	85.7	90.8	95.4	73.1	58.6	64.6	74.6	63	34.3	14.5

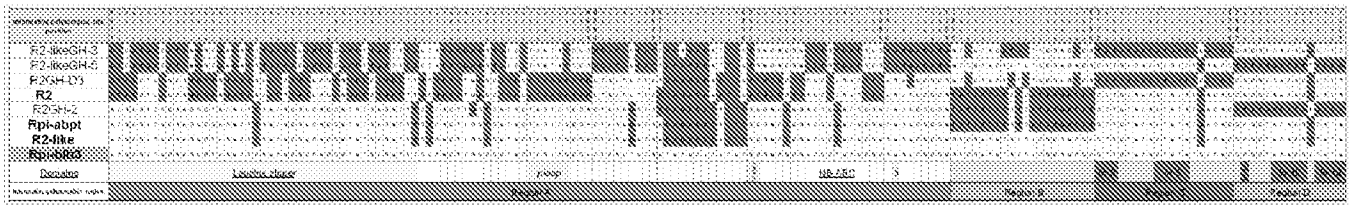


Figure S2: Nucleotide polymorphism analysis of *Rpi-blb3* and 7 homologues sharing informative polymorphic or homologous sequences. In bold are the functional genes *R2*, *Rpi-abpt*, *R2-like*, *Rpi-blb3*. Only informative polymorphic sites are shown, which reduces the sequence size from 2444 bp to 170 bp. Highlighted in red are the positions polymorphic to *Rpi-blb3* sequence. The vertical number at the top of each row indicates the corresponding nucleotide position in the full consensus sequence, the limits of the recombination regions positioned on the bottom of the figure are indicated in bold. The positions of the different domains characterizing LZ-NBS-LRR genes and the informative polymorphic regions A to D are indicated down to the sequences.

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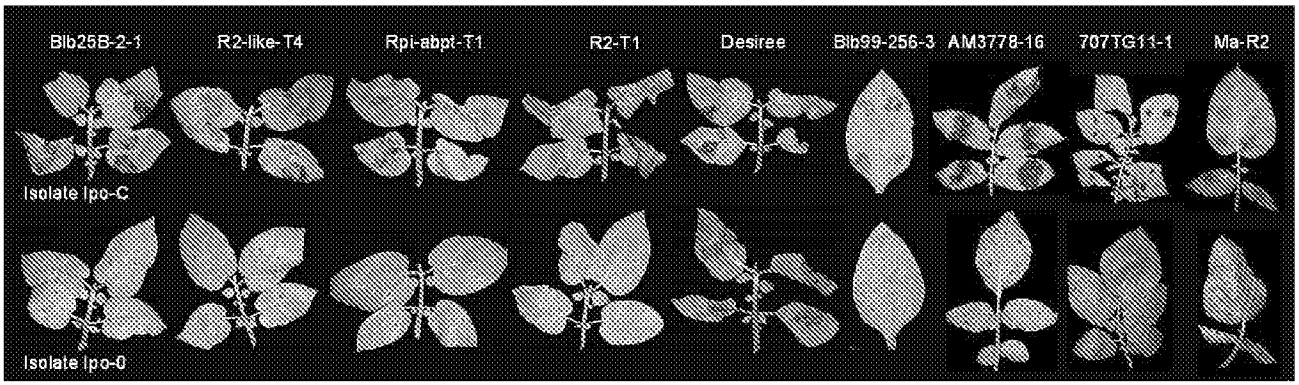


Figure S3: Genetic complementation for late blight resistance. Six days after inoculation with *Phytophtora infestans* isolate IPO-0, the hypersensitive response (HR) was observed for the positive control Blb25B-2-1 carrying *Rpi-blb3*, the transformants expressing *R2*-like (R2-like-T4), *Rpi-abpt* (Rpi-abpt-T1), *R2* (R2-T1) and the parental clones harboring *Rpi-blb3* (Blb99-256-3), *R2-like* (AM3778-16), *Rpi-abpt* (707TG11) and *R2* (MaR2); large necrotic lesions and massive sporulation were noted for potato cultivar Desiree. All the tested leaves were susceptible to the virulent isolate IPO-C.

DISCUSSION

In this study we describe the cloning and functional characterization of four homologous *R* genes from the major late blight resistance locus on LG IV of potato. Previously, marker order and allelic conservation suggested that *Rpi-blb3, Rpi-abpt, R2* and *R2-like* reside at the same locus on LG IV (Park et al. 2005a, 2005b, 2005c). *Rpi-blb3* and *Rpi-abpt* were cloned using a map-based cloning approach whereby physical maps of both loci were generated by screening appropriate BAC-libraries with a highly linked molecular marker, followed by subsequent rescreening of the libraries with novel BAC-end markers until the outer ends of the BAC-contigs exceeded the genetic boundaries of the *R* loci. In the case of *Rpi-blb3,* two candidate NBS-LRR genes were identified and subcloned into a binary vector and targeted for complementation analysis through stable transformation of a susceptible potato variety.

For *Rpi-abpt,* a NBS-LRR candidate was cloned from the BAC using a homology-based PCR cloning strategy using primers designed on the functional *Rpi-blb3* gene. In addition complementation was carried out using an *Agrobacterium*-mediated transient expression assay in *N. benthamiana* followed by detached leaf assays with appropriate *P. infestans* isolates (Rietman et al. in preparation).

Development of the transient complementation assay together with the successful *in planta* expression of *Rpi-abpt* and other *R* genes (Rietman et al. in preparation) using the DNA regulatory elements of *Rpi-blb3*, facilitated for the rapid cloning of *R2* and *R2-like* through an efficient functional allele mining strategy. A prerequisite for such an allele mining strategy is prior knowledge of candidate gene sequences at the locus of interest. Observations within the *Solanaceae* indicate that *R* gene clusters are conserved across species, thus enabling comparative genomics (Grube et al. 2000; Huang. 2005; van der Vossen et al. 2005). As more and more *R* genes are mapped and cloned, the greater the chance that novel *R* genes reside at known and well-characterized loci. Moreover, ongoing potato and tomato genome sequencing projects by international consortia promise to provide a complete survey of the distribution of *R* gene clusters in the *Solanaceae,* enabling even faster cloning of *R* genes through allele mining strategies.

Interestingly, with respect to known functional R proteins, Rpi-blb3, Rpi-abpt, R2, and R2-like share the highest similarity (34.9% aa) with RPP13 from *A. thaliana* (Bittner-Eddy et al. 2000), which confers resistance to *H. arabidopsidis*. Mining of *RPP13* alleles in a diverse set of *A. thaliana* accessions revealed both extreme sequence diversity and functional diversity in pathogen recognition (Rose et al. 2004). In line with this observation a significant excess of amino acid polymorphism was

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localized within the LRR domain of *RPP13*. In the case of *Rpi-blb3, Rpi-abpt, R2,* and *R2-like*, the LRR domains are nearly identical despite significant polymorphism at the CC and NBS domains, suggesting conservation of function, and indeed we have shown that all four proteins interact with the same effector protein, PiAVR2, which was recently cloned through a map-based cloning approach (personal communication, E. Gilroy). Although the origin of *Rpi-abpt* and *R2-like* remain unclear, this finding is a second example of how *R* gene recognition specificities can be conserved across *Solanum* species originated from different potatoe superseries, in this case *S. bulbocastanum* (*Rpi-blb3*) belongs to the *Potatoe Stellata Bulbocastana* and *S. demissium* (*R2*) to the *Potatoe Rotata demissa*. Recently we demonstrated both gene conservation and specificity conservation between the *S. bulbocastanum*-derived gene *Rpi-blb1* and the *S. stoloniferum* and *S. papita*-derived orthologues *Rpi-sto1* and *Rpi-pta1,* respectively (Vleeshouwers et al. 2008). Despite apparent positive selection in the LRR domain, the LZ and NBS domains are more polymorphic, those of R2 being the most divergent. Clear blocks of sequence affiliation between the four functional R proteins and additional *Rpi-blb3* gene homologs, suggest exchange of LZ, NBS and LRR domains, and also of specific LRRs, underlining the modular nature of these proteins. *R2* has a particular gene structure likely to be the product of a relatively recent recombination event. The LZ-NBS domain (nucleotides 1-1149) is highly affiliated to that of the non-functional *R2-GH-D3*, whereas the LRR domain (nucleotides 1059-2544) is highly affiliated to that of *Rpi-abpt* and the non-functional *R2-GH-65*, revealing a sequence exchange track between nucleotides 1059 and 1149. Sequence exchange between RGHs has been proposed as a major mechanism shaping *R* gene diversity in plants (Michelmore and Meyers 1998; Meyers et al. 1999; Kuang et al. 2004). Interestingly, the approximate position of this recombination site corresponds to the one described by Rose et al. (2004) for the *RPP13* allele *ksk*-2, a region between the conserved GLPL and VHD motifs of the NB-ARC domain. This is in line with the expectation that in multi-domain proteins, sequence exchange tracks will be concentrated at specific sites that separate domains with independent functions.

Preliminary results from ongoing *Rpi-blb3* allele mining studies in *Solanum* germplasm indicate that *Rpi-blb3* is present in several other species, e.g. *S. brachistotrichum* and *S. pinnatisectum*. In addition several *Rpi* genes recently identified in our laboratory (data not shown) also map to the *Rpi-blb3* interval. The QTLs for late blight resistance (Pi-QTL) (Sandbrink et al 2000) explaining 40% of the phenotypic variation, was mapped to the *Rpi-blb3* locus, and renamed *Rpi-mcd1* (Tan et al. 2008). Similarly, high resolution mapping data of the major Pi-QTL identified in cv. Stirling strongly suggest that this Pi-QTL is also an *Rpi-blb3* homologue (Hein et al. 2007; Ingo Hein, personal communication). And various other examples of Pi-QTLs on the short arm of LG IV have been described (Leonards-Schippers et al. 1994; Sandbrink et al. 2000; Bradshaw et al. 2004; Bradshaw et al. 2006).

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Rpi-blb3, Rpi-abpt, R2 and R2-like specifically detect PiAVR2. Do the Rpi-blb3 homologues putatively underlying the Pi-QTL described above also recognize PiAVR2 homologues? If so do they weekly recognize an allele, which is conserved throughout *P. infestans* or do they recognize multiple alleles, thus resulting in non-race-specific resistance?

Current models for NB-LRR R proteins suggest a dual role for the LRR domain, not only as recognition specificity determinant but also as a repressor of inappropriate NB activation (Belkhadir et al. 2004). Furthermore, evolutionary analyses of R proteins and their structure has shown selection pressure on the LRR region but also on other domains within R proteins, which, in light of the dual function, may point towards co evolution in R proteins to establish and maintain recognition of effectors while maintaining the inhibitory function (McDowell and Simon. 2006). Our findings are slightly different, contradictory, indicating conservation in the LRR domain but more variation in the NBS domain, that may point towards different signaling pathways or additional effectors being recognized by these R proteins.

MATERIALS AND METHODS

Plant material and *Phytophthora infestans isolates*

In this study we used the four resistant parental clones Blb99-256-3 harbouring *Rpi-blb3* (Park et al., 2005a), 707TG11-1 *Rpi-abpt* (Park et al., 2005c), CEBECO44158-4 (MaR2) *R2* and AM3778-16 *R2-like*, respectively (Park et al.2005b). F1 progeny plants of population RH4x103 were derived from a cross between the late blight resistant clone 707TG11-1 and the diploid potato clone RH89-039-16 (Park et al., 2005c).The susceptible potato cultivar Desiree was used for *Agrobacterium*-mediated transformation. The characteristics and origin of *P. infestans* isolates used in this study are indicated in supplementary Table S3.

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Supplementary Table S3 Characteristics of *P. infestans* isolates used in this study. Resistance genes which didn't have a clear phenotype from one repeat to the other are in brackets in the virulence profile.

Isolate	Year	Geographic origin	Mating type	Obtained from	Virulence profile
90128	1990	Geldrop, Netherlands	A2	Govers, Phytopathology WUR	1.3.4.7.8.10.11
H30P04	unknown	Artificial hybrid	unknown	Govers, Phytopathology WUR	3a, 7, 10
USA618	n.d	Toluca Valley, Mexico	A2	Fry, Cornell, USA	1.2.3.6.7.10.11
89148-09	1989	Netherlands	A1	Govers, Phytopathology WUR	0
IPO-0	n.d	Netherlands	n.d	Kessel, PRI, WUR	0
IPO-C	1982	Belgium	A2	Kessel, PRI, WUR	1.2.3.4.5.6.7.10.11
VK98014	1998	Veenkolonién, Netherlands	A1	Kessel, PRI, WUR	1.(2).4.11
Dinteloord	n.d	Dinteloord, Netherlands	n.d	Kessel, PRI, WUR	1.(2).4
EC1	n.d	Ecuador	n.d	Birch, SCRI, Scotland	1.3.4.7.10.11
F95573	1995	Flevoland, Netherlands	A1	Govers, Phytopathology, WUR	1.3.4.7.10.11
IPO-428-2	1992	Ede, Netherlands	A2	Kessel, PRI, WUR	1.3.4.7.8.10.11
Katshaar	n.d	Katshaar, Netherlands	n.d	Kessel, PRI, WUR	1.3.4.7.10.11
PIC 99177	1999	Metepec, Mexico	A2	Kessel, PRI, WUR)	1.2.3.4.7.11
PIC 99183	1999	Metepec, Mexico	A2	Kessel, PRI, WUR	1.3.4.5.7.8.10.11
PIC 99189	1999	Metepec, Mexico	A2	Kessel, PRI, WUR	1.2.5.7.10.11
88069	1988	Netherlands	A1	Govers, Phytopathology, WUR	1.3.4.7
PY23*	1999	GGO (modified 88069)	A1	Govers, Phytopathology, WUR	1.3.4.7

* References: Kamoun et al. 1998; West et al. 199

BAC library construction

Clones Blb99-256-3 and 707TG11-1 were used as DNA sources for the construction of BAC libraries. High-molecular weight DNA preparation and BAC library construction were carried out as described previously (Rouppe van der Voort et al. 1999). Approximately 74000 clones with an average insert size of 85 kb, corresponding to 8 genome equivalents, were obtained per library. The BAC clones were stored as bacterial pools containing approximately 700 to 1000 white colonies. These were generated by scraping the colonies from the agar plates into LB medium containing 18% glycerol and 12.5 µg ml^{-1} chloramphenicol using a sterile glass spreader. These so-called super pools were stored at -80ºC. Marker screening of the BAC libraries was done, first by isolating plasmid DNA from each pool using the standard alkaline lysis protocol and PCR was carried out to identify positive pools. Bacteria corresponding to positive pools were diluted and plated on LB agar plate containing chloramphenicol (12.5 µg ml^{-1}). Individual white colonies were picked into 384-well microtitre plates and single positive BAC clones were subsequently identified by marker screening as described by Rouppe van der Voort (1999). Names of BAC clones isolated from the super pools carry the prefix Blb (e.g. Blb25) or TG (e.g. TG9).

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Subcloning of candidate genes

Candidate RGAs were subcloned from BAC clone BLB25 and TG9 as follows. Aliquots of approximately 1 µg BAC DNA were digested with 0.03U of *Sau3A*l restriction enzyme for 10 min. The partially digested BAC DNA was subjected to electrophoresis at room temperature in 0.5X TAE using a linear increasing pulse time of 1-10 sec and a field strength of 90 V cm^{-1} for 6 h. After electrophoresis, the agarose gel was stained with ethidium bromide to locate the region of the gel containing DNA fragments of approximately 10 Kbp in size. This region was excised from the gel and treated with GELASE (Epicentre Technologies, Madison, WI, USA) according to the manufacturer's instructions. The size-selected DNA was ligated to the *BamH*I-digested and dephosphorylated binary vector pBINPLUS (van Engelen et al. 1995) followed by transformation to ElectroMAX E.coli DH10B competent cells (Life technologies, Paisley, UK).

DNA sequencing and computer analysis

BAC clone sequencing was carried out using a shotgun cloning strategy. Sequencing reactions were performed using a dye terminator cycle sequencing reaction kit (Perkin-Elmer, Pt Biosystem, Warrington, UK), M13 universal forward and reverse primers, and an ABI377 automated sequencer (Applied Biosystem, La Jolla, CA, USA). Sequence contigs were assembled using the STADEN sequence and analysis program (Dear and Staden 1991).

The binary constructs were sequenced using a primer walking strategy (700 bp by 700 bp). Gene structures were predicted using FGENESH++ (Softberry) and protein sequences were deduced by translation of ORF using the standard genetic code. Multiple sequence alignments were conducted using CLUSTALX 1.81 (Thompson et al. 1997). The search for genes homologous to *Rpi-blb3* was carried out using the Basic Local Alignment Search Tool (BLAST). Conserved domains were identified using Swiss-Prot (InterProScan, EMBL-EBI, ExPASy, SAPS).

Resistance assay

Detached leaf assays were used to determine the resistance phenotypes of primary transformants of potato and transiently transformed *N. benthamiana* leaves. For complementation analyses, primary transformants were tested with isolates IPO-0 and 90128 (Fig. 2 and Table S3). Inoculum preparations and inoculations were performed as described by Vleeshouwers et al. (1999). Six days after inoculation, plant phenotypes were determined. Leaves showing no symptoms or a localized necrosis at

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the point of inoculation were scored as resistant and those with clear sporulating lesions (visible by naked eye) as susceptible.

Transient complementation in N. benthamiana

Infiltrations of *Agrobacterium* strains carrying the candidate RGH were performed in *N. benthamiana.* Recombinant COR308 *A. tumefaciens* cultures were grown in LB medium (10 gram bacteriological peptone, 10 gram NaCl and 5 gram yeast extract in 1 liter milli-Q water) supplemented with 5 mg/L tetracycline and 50 mg/L kanamycin. After one or two days a calculated amount of culture (according to OD 0.5 at 600 nm) was transferred to YEB medium (5 gram beef extract, 5 gram bacteriological peptone, 5 gram sucrose, 1 gram yeast extract, 2ml 1 M MgSO4 in 1 liter MQ water) supplemented with kanamycin for all strains. After 1 day overnight cells were centrifuged at 3500 rpm and re-suspended in MMA medium (20 gram sucrose, 5 gram MS salts and 1.95 gram MES) supplemented with 1 ml 200 mM acetosyringone to a final OD of 0.2 and infiltrated into leaves of 4 weeks old plants with a 3ml syringe. After two days, infiltrated leaves were detached and inoculated with *P. infestans* strains IPO-C (Table S3) and PY23 in detached leaf assays. Hypersensitive response (HR) or *P. infestans* sporulation was observed between 4 to 8 days post inoculation.

Agrobacterium-mediated coexpression in N. benthamiana

pBP-Rpiblb3, *pKGW-Rpi-blb3*, *pKGW-Rpi-abpt*, *pKGW-R2*, *pKGW-R2-like*, *pKGW-Rpi-sto1*, *pBP-abptGH-A* and *pBP-R3a* were transferred to the *Agrobacterium tumefaciens* strain AGL1 (Lazo et al. 1991) containing the helper plasmid pVirG and pSoup (van der Fits et al. 2000). Recombinant *A. tumefaciens* cultures were grown in LB medium supplemented with 5 mg/L tetracycline , 100 mg/L carbeniciline, 75 mg/L choramphenicol and 50 mg/L Kanamycin. The avirulence effectors were cloned into the pGRAB vector (kindly supplied by Sean Chapman, SCRI. Scotland. UK) and transferred to the same *A. tumefaciens* strain AGL1. Recombinant *A. tumefaciens* cultures were grown in LB medium supplemented with 30 mg/L choramphenicol and 50 mg/L kanamycin. The same protocol as the transient complementation was used for the sub-cultures. *PiAVR Agrobacterium* culture and *Rpi-gene Agrobacterium* culture were mixed at a final OD6oo of 0.1 for each and infiltrated into leaves from 5 to 7 week old plants. Specific recognition of the *AVR* gene by the *R* gene induces hypersensitive cell death response (HR) from 5 days post-infiltration.

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Transformation of susceptible potato variety

Binary plasmids harboring the candidate genes *Blb3GH-A*, *Blb3GH-B*, *AbptGH-A* and *AbptGH-B* were transferred to *Agrobacterium tumefaciens* strain COR308 (Hamilton et al. 1996) and the constructs pKGW-Rpi-abpt, pKGW-R2-like, pKGW-R2 in AGL1 (Lazo et al. 1991). After verifying their stability in *Agrobacterium* these clones were transformed to the susceptible potato variety Desiree. Overnight cultures of the transformed *A.tumefaciens* strain were used to transform internodal cuttings from *in vitro* grown plants (Heilersig et al. 2006). A total of 100 to 200 explants were used for each transformation. Three to eight plants per transformant were transferred to the greenhouse.

Allele mining

Primers of 32 nucleotides were designed on Rpi-blb3 sequence, with the forward primer beginning at the start codon (Blb3-start) and the reverse primer beginning at the stop codon (Blb3-end; Table 1). The BAC clone TG92 containing *Rpi-abpt* and the genomic DNA of the parental resistant genotype AM-(3778-16) containing R2-like, were used as template in a long range PCR reaction (95 C: 2'40'', 30X [94 C: 20'', 56.8 C: 25'', 64.3 C:7'], 64.3 C: 25') using the high fidelity DNA polymerase *PfuTurbo*® (Stratagene, la Jolla, CA, U.S.A). PCR products were separated on agarose gel and purified using the QIAquick Gel Extraction Kit from Qiagen (Hilden, Germany). The purified pool of R2-likeGH ORFs and the purified Rpi-abpt ORF were used in a BP reaction together with the donor plasmid pDONR 221 according to the protocol described by (Untergasser 2006b). Subsequently, the BP reaction products were transferred to DH10B *E. coli* competent cells and plated on LB-agar plates containing the antibiotic kanamycin at the concentration of 100 µg/ml. Colonies were cultured in LB liquid medium supplemented with kanamycin. Plasmid DNA was subsequently extracted by mini-prep protocol adapted from Sambrook et al (2nd edition, 1989) using the P1, P2, P3 solutions from Qiagen. The first and last 700 bp of plasmids containing R2-likeGH ORFs were sequenced and classified. Then one clone of each group was entirely sequenced and compared to the *Rpi-blb3* sequence. The clones sharing high similarity with *Rpi-blb3* were cloned into the destination vector pKGW-MG (kindly provided by Andreas Untergasser) in between *Rpi-blb3* regulatory elements via a multiple LR reaction with the *Rpi-blb3* promotor in pDONR-P4P1R, the *Rpi-blb3* terminator in pDONR-P2RP3, the ORF of interest in pDONR221, and the binary destination, using the protocol of (Untergasser 2006a). The final expression constructs were then transferred to *A. tumefaciens* strain COR308 after the configuration of the constructs was confirmed by PCR.

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Supplementary Table S1: Genebank accessions numbers

Gene	accession number
Rpi-blb3	FJ536346
R2-like	FJ536323
Rpi-abpt	FJ536324
R2	FJ536325
blb3GH-A	FJ536326
R2-likeGH-2	FJ536327
R2-likeGH-3	FJ536328
R2-likeGH-5	FJ536329
abptGH-A	FJ536330
R2GH-2	FJ536331
R2GH-3	FJ536332
R2GH-5	FJ536333
R2GH-7	FJ536334
R2GH-8	FJ536335
R2GH-9	FJ536336
R2GH-10	FJ536337
R2GH-11	FJ536338
R2GH-12	FJ536339
R2GH-13	FJ536340
R2GH-14	FJ536341
R2GH-15	FJ536342
R2GH-16	FJ536343
R2GH-D3	FJ536344
R2GH-65	FJ536345

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Chapter 3

Diversity, distribution and evolution of *Solanum bulbocastanum* Late Blight resistance genes

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ABSTRACT

Knowledge on the evolution and distribution of Late Blight resistance genes is important for a better understanding of the dynamics of these genes in nature. We analyzed the presence and allelic diversity of the Late Blight resistance genes, *Rpi-blb1*, *Rpi-blb2* and *Rpi-blb3*, originating from *Solanum bulbocastanum* in a set of tuber-bearing *Solanum* species. These genes were only present in some Mexican diploid as well as polyploid species closely related to *S. bulbocastanum*. Sequence analysis of the fragments obtained from the *Rpi-blb1* and *Rpi-blb3* gene suggests an evolution through recombination and point mutations. For *Rpi-blb2* only sequences identical to the cloned gene were present in *S. bulbocastanum* accessions, suggesting that it has emerged recently. The three resistance genes occurred in different combinations and frequencies in *S. bulbocastanum* accessions and their spread is confined to Central America. A selected set of genotypes was tested for their response to the avirulence effectors IPIO-2, Avr-blb2 and Pi-Avr2, which interact with Rpi-blb1, Rpi-blb2 and Rpi-blb3, respectively, as well as by disease assays with a diverse set of isolates. Using this approach some accessions could be identified that contain novel, yet unknown, Late Blight resistance factors that in addition to the *Rpi-blb1*, *Rpi-blb2* and *Rpi-blb3* genes.

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INTRODUCTION

Solanum bulbocastanum is well known for its resistance to Late Blight (Niederhauser and Mills 1953), a disease caused by the oomycete *Phytophthora infestans*. The species belongs to the section *Petota* of the genus *Solanum*. It is a diploid, self-incompatible species native to Central America. It inhabits mountainous areas at an altitude between 1200-2300 m (Spooner et al. 2004) from northwestern Mexico (Durango and Nayarit regions) all the way south to Honduras..

Although characterized by Late Blight resistance, *S. bulbocastanum* resistance sources have not been widely exploited yet, which is mainly due to crossing barriers with cultivated potato (Helgeson et al. 1998; Hermsen and Ramanna 1973). Until now, 4 different NBS-LRR resistance genes have been identified in and cloned from *S. bulbocastanum*, namely *Rpi-Blb1* (van der Vossen et al. 2003) also known as *RB* (Song et al. 2003), *Rpi-Blb2* (van der Vossen et al. 2005) *Rpi-Blb3* (Lokossou et al. 2009) and *Rpibt1* (Oosumi et al. 2009), which opens the possibility to study the distribution and diversity of these genes in the section *Petota.*

The distribution of specific resistance genes in natural populations has been studied in a few cases only, *Rps2* and *Rpp13* in *Arabidopsis thaliana* (Mauricio et al. 2003; Rose et al. 2004), and *Cf-4* and *Cf-9* in tomato (Kruijt et al. 2005; Caicedo 2008) . Mauricio et al. (2003) studied the diversity of *Rps2*, a resistance gene against the bacteria *Pseudomonas syringae*, in a world wide sample of 27 *A. thaliana* accessions. They favored balancing selection as the explanation for the *Rps2* variation. This maintenance of variance by natural selection might be a general feature for the dynamics of *R*-gene evolution (Ellis et al. 1999; Luck et al. 2000). From the study on diversity for *Rpp13*, a resistance gene in *A. thaliana* against *Hyaloperonospora arabidopsis,* which is characterized by an extreme sequence diversity and resistance to various pathogen isolates; the suggestion was made that the co-evolutionary interactions between host and pathogen must be very active (Rose et al. 2004). Confirmation was provided with the analysis of the recently identified *Avr*-gene of *Rpp13*, *Atr13*, which reveals high sequence diversity for this effector as well (Allen et al. 2008) and moreover, one *Rpp13* allele seems to recognize another *H. arabidopsis* effector (Hall et al. 2009). Kruijt et al. (2005) showed that a high proportion of wild tomato plants specifically recognize the *C. fulvum* Avr4 and Avr9 elicitors, and functional homologs of the *C. fulvum* resistance genes *Cf-4* and *Cf-9* are conserved in diverged tomato species. The *Cf-4* and *Cf-9* genes appear to have been maintained by selection pressure imposed by the pathogen *Cladosporium fulvum*. Caicedo (2008) studied the distribution of *Cf-2* homologs in natural populations of *S. pimpinellifolium* and deduced that primarily the geographic distribution of *Cf-2* homolog diversity has been shaped by demographic factors or by selective pressure with a clinal geographic distribution. Levels of homologue diversity varied greatly between populations.

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As population genetic processes and dynamics might affect the evolution of *R*-genes, a better understanding of variation within and between populations is crucial. Spooner (2009) evaluated several factors that might be predictive for identifying pest and disease resistant plants in wild relatives of the cultivated potato. For late blight resistance they identified taxonomic, climatic and geographic (latitude and altitude) variables. From these analyses it was clear that species level was the best predictor for resistance against *P. infestans*. These results stressed the need to use more focused evaluations of germplasm and to have a clear picture of geographical patterns of diversity in Late Blight resistance genes in wild *Solanum* species. To analyze these, here, use has been made of well studied *R*-genes like *Rpi-blb1, Rpi-blb2* and *Rpi-blb3* which are terrific candidates since both *Avr* and *R*-genes have been identified and cloned.

The *Rpi-blb1* is located on linkage group VIII, near marker CT64 in a cluster of 4 Resistance Gene Analogues (RGA's) (van der Vossen et al. 2003). Its susceptible haplotype, rb, is characterized by an 18 bp deletion (Song et al. 2003). In an evaluation by Wang et al. (2008) using 44 species of *Solanum* section *Petota* for *Rpi-blb1* (*RGA2*) and its flanking *RGA's* (*RGA1* and *RGA3*) it was shown that the genes had a different distribution and diversity. The flanking *RGA1* appeared to be highly conserved within the tuber bearing *Petota* and was also found in the non tuber bearing species of the section, while *RGA3* was only present in some tuber bearing species. The *Rpi-blb1* gene was found in Mexican diploid species (*S. bulbocastanum* and *S. cardiophyllum*) and the Mexican polyploid species *S. stoloniferum* (including *S. papita* and *S. polytrichon*) (Wang et al. 2008). Functional analysis of the genes found in *S. stoloniferum* (*Rpi-sto1* and *Rpi-pta1*) indeed confirmed the functional homology with *Rpi-blb1* (Vleeshouwers et al. 2008). The occurrence of *Rpi-blb1* homologues in *S. stoloniferum* was suggested to be the result from common ancestry, e.g. *S. bulbocastanum* was suggested as one of the parents of the allopolyploid *S. stoloniferum* (Wang et al. 2008).

Rpi-blb2 is located on linkage group VI near marker CT119, in a locus harboring at least 15 *Mi-1* gene homologues (van der Vossen et al. 2005). The *Mi-1* gene of tomato confers resistance to the root knot nematode *Meloidogyne incognita* (Milligan et al. 1998) and shares 82% overall protein sequence identity with *Rpi-blb2* (van der Vossen et al. 2005). *Rpi-ver1,* a Late Blight *R*-gene from *S. verrucosum* also maps in this region (Jacobs et al. 2010). Other *R*-genes found at this locus in tomato include *Ol-1* and *Ol-3* (Bai et al. 2005), *Cf2* and *Cf5* (Dickinson et al. 1993), conferring resistance to *Oidium neolycopersici* and *Cladosporium fulvum,* respectively. Apart from *S. bulbocastanum*, no other species were found to contain *Rpi-blb2* (Wang et al. 2008).

Rpi-blb3 is located on linkage group IV, near marker TG339 in a major Late Blight resistance gene cluster containing *R2*, *Rpi-abpt* and *R2-like* (Park et al. 2005). Also Late Blight resistances with a

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more QTL-like appearance like those of *S. microdontum* (Sandbrink et al. 2000; Tan et al. 2008) and those found in tomato (Brouwer et al. 2004) map into the same region. Tan et al. (2008) proposed that the QTL from *S. microdontum,* indentified as *Rpi-mcd1,* is a major *R*-gene. Other pathogen *R*-genes, mapping to the same region, include Ny_{tbr} conferring resistance to *Potato virus Y* (Celebi-Toprak et al. 2002) and the *Hero* resistance gene which confers resistance to all known pathotypes of *Globodera rostochiensis* and partial resistance to *Globodera pallida* (Ernst et al. 2002). Allele mining studies for *Rpi-blb3* are not yet reported.

Distribution of *R*-genes in natural populations is not easy to study phenotypically, as resistance to a particular pathogen might result from the presence of one or more different *R*- genes, which is the case for populations of *S. bulbocastanum*. One approach could be to use *Avr*-genes to determine whether a corresponding *R*-gene is present, in our case Avrblb1, which interacts with Rpi-blb1 (Vleeshouwers et al. 2008), Avrblb2 interacting with Rpi-blb2 (Oh et al. 2009) and Pi-Avr2 interacting with Rpi-blb3 (Lokossou et al. 2009). However the *Rpi-blb3* gene has several close relatives, all recognizing the same set of *Avr*-genes (Lokossou et al. 2009). Also carrying out such analysis in a large number of different species is not straight forward. Studying the distribution of *R*-genes with highly specific molecular markers and deduction of the phenotype for positive genotypes is an alternative. Still, one has to keep in mind that the functionality of *R*-genes might be changed by one single nucleotide polymorphism (SNP) or by insertions/deletions. A combination of the two approaches and screening with key *P. infestans* isolates seems a beneficial strategy as that will also allow the identification of other resistance genes towards this devastating disease.

In this paper, we studied the allelic frequencies of the *Rpi-blb1*, *Rpi-blb2* and *Rpi-blb3* genes in several wild tuber-bearing *Solanum* accessions by performing allele mining with *R*-gene-specific primers. Additionally, Avr expression assays as well as detached leaf assays with key *P. infestans* isolates were utilized to strengthen the evidence for the presence of functional alleles. We relate the occurrence of the genes to the geographical origin of the accessions.

RESULTS

Screening of Solanum section Petota for the presence of Rpi-blb resistance genes

A total of 196 different taxa from *Solanum* section *Petota* were screened for the presence of the *Rpi-blb1* and *Rpi-blb3* genes. The presence of *Rpi-blb2* was not assessed in this wide set of taxa as a

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previous study had already shown that the distribution of this gene was confined to *S. bulbocastanum* (Wang et al. 2008). We used the plant material described by Jacobs et al (2008) and as far as available at least 5 accessions from each species and 5 individuals per accession (in total 4929 genotypes). One screen was carried out with *Rpi-blb1* recognizing 1/1' primer pair, which is specific for the functional allele of *Rpi-blb1* (Colton *et al.* 2006). PCR fragments were detected in *S. bulbocastanum*, *S. cardiophyllum* ssp. *cardiophyllum* and *S. stoloniferum* (sensu Spooner et al. 2004) only.

The collection was also screened with primers specific for *Rpi-blb3* and 6 species were found to contain putative *Rpi-blb3* homologues: *S. pinnatisectum*, *S. bulbocastanum* (including some subspecies), *S. hjertingii, S. nayaritense, S. brachistotrichum* and *S. stoloniferum*.

Detailed investigation of S. bulbocastanum and related species

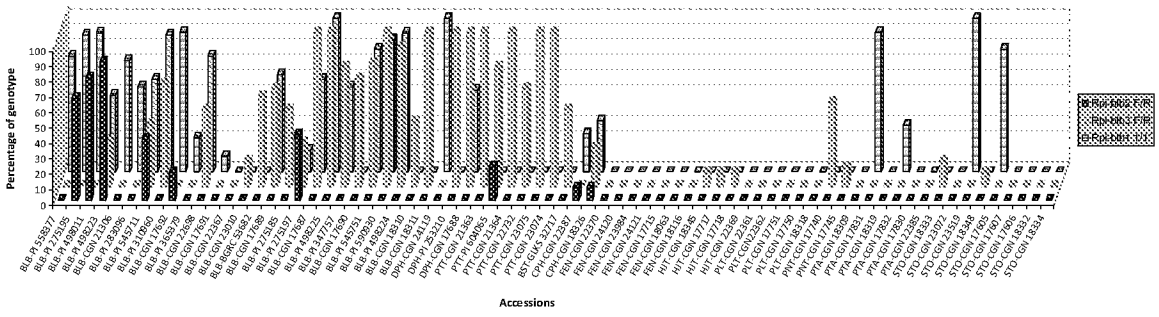
An additional 72 accessions originating from Mexico and Guatemala of the series *Bulbocastana*, *Pinnatisecta* and *Longipedicellata* was evaluated for the presence of *Rpi-blb1*, *Rpi-blb2* and *Rpi-blb3* genes. For 63 accessions precise coordinates (longitude, latitude data) were available, pointing to 52 different locations (Table S1).

The presence or absence of *Rpi-blb1* was analyzed using the primer combinations Blb1 F/R, detecting the *Rpi-blb1* locus (Wang et al. 2008) and Blb 1/1' (Colton et al. 2006). The RGA1 F/R primer pair served as a positive control for DNA quality as this gene is present in all tuber-bearing *Solanaceae* (Wang et al. 2008).

The primer pair Blb1 F/R amplified fragments of the expected size in 27 *S. bulbocastanum*, 1 *S. brachistotrichum*, 2 *S. cardiophyllum*, and 4 *S. stoloniferum* accessions. The primer pair Blb 1/1' amplified fragments in a total of 29 accessions from 23 different locations and composed of 23 accessions of *S. bulbocastanum*, 2 of *S. cardiophyllum*, and 4 of *S. stoloniferum* (Table S1 and Figure 1A).

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A.



B.

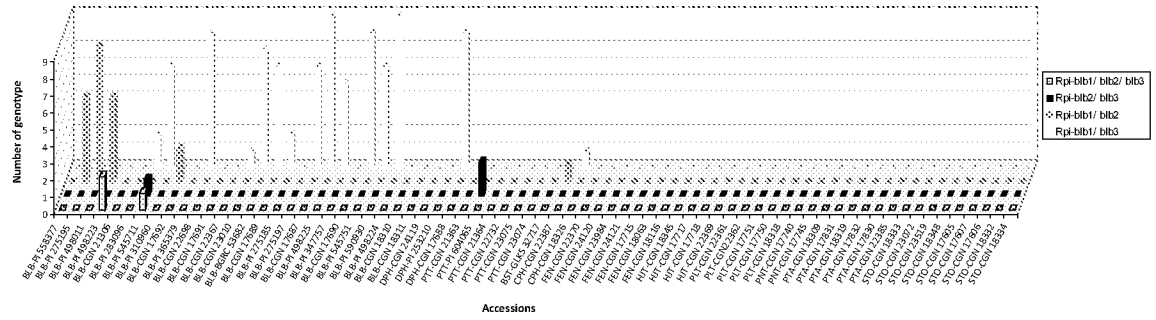


Figure 1:*Rpi-blb1*, *Rpi-blb2* and *Rpi-blb3* presence in *S.bulbocastanum* and related species. (A) Percentage of genotype per accessions containing *Rpi-blb* genes fragments. (B) Number of genotypes per accessions containing combinations of *Rpi-blb* genes fragments. Abbreviations: BLB: *S. bulbocastanum*, BST: *S. brachistotrichum*, CPH: *S. cardiophyllum*, DPH: *S. bulbocastanum sbsp dolichophyllum*, FEN: *S. fendleri*, HJT: *S. hjertingii*, PLT: *S. polytrichon*, PNT: *S. pinnatissectum*, PTA: *S. papita*, PTT: *S. bulbocastanum* subsp. *partitum*, STO: *S. stoloniferum.*

Supplementary Table S1: List of the accessions used to identify the frequency of the three *S. bulbocastanum* genes *Rpi-blb1*, *Rpi-blb2*, *Rpi-blb3*. From the left to the right the columns report the plant taxon, the accession number, the country of origin, the location coordinates (latitude-longitude in degrees and minutes); the total number of genotype tested positive by PCR with specific primers to *Rpi-blb1*, *Rpi-blb2*, *Rpi-blb3*, the haplotypes identified after sequencing; the number of genotype per accession which contain the different combination of genes *Rpi-blb1+Rpi-blb2*, *Rpi-blb1+Rpi-blb3*, *Rpi-blb2+Rpi-blb3*, *Rpi-blb1+Rpi-blb2+ Rpi-blb3*.

S1a. *S. bulbocastanum* accessions

Taxon	Accession number	Country	Coordinates		Number of genotypes	Rpi-blb genes identification								Rpi-blb genes Combination			
			lat	lon		RGA1	Blb1 F/R	Blb1 1/1´	Haplotype	Blb2 F/R	Haplotype	Blb3 F/R	Haplotype	blb1/ blb2	blb1/ blb3	blb2/ blb3	blb1/ blb2/ blb3
BLB	PI 558377	MEX	16.16N	96.41W	8	8	7	6	A6, A7	-		-		-	-	-	-
BLB	PI 275195	MEX	17.02N	96.46W	9	9	8	8	A1, A3	6	A	-		**5**	-	-	-
BLB	PI 498011	MEX	17.02N	96.46W	10	10	9	9	A3, C7	8	A	-		**8**	-	-	-
BLB	PI 498223	MEX	17.02N	96.46W	10	10	6	5	A9, A10, C2	9	A	3	A1	**5**	-	-	**2**
BLB	CGN 21306	MEX	17.30N	96.27W	11	11	8	8	A1, A2, A3	-		-		-	-	-	-
BLB	PI 283096	MEX	17.30N	96.52W	9	9	7	5	A4	-		-		-	-	-	-
BLB	PI 545711	MEX	17.33N	99.30W	10	10	6	6	D2, D3	4	A	4	A2		**2**	**1**	**1**
BLB	PI 310960	MEX	17.49N (b)	98.08W	9	9	8	8	H1, I1	-		6	A2	-	**6**	-	-
BLB	CGN 17692	MEX	17.53N	96.33W	11	11	10	10	A2, B1	2	A	-		**2**	-	-	-
BLB	PI 365379	MEX	18.43N	97.19W	9	9	2	2	A8	-		-		-	-	-	-
BLB	CGN 22698	MEX	19.11N	98.13W	16	12	12	12	A1, C2	-		8	A2	-	**8**	-	-
BLB	CGN 17691	MEX	19.13N	98.48W	10	9	5	1	A1, M1	-		-		-	-		-
BLB	CGN 22367	MEX	19.13N	98.48W	10	10	10	-	M1	-		-		-	-	-	-
BLB	CGN 23010	MEX	ND	ND	12	10	8	-	E2, M1	-		2	A2	-	**1**	-	-
BLB	BGRC 53682	MEX	19.15N (a)	98.53W	24	18	11	-	A5, M1	-		14	A2	-	**7**	-	-
BLB	CGN 17689	MEX	19.21N	99.12W	11	11	11	7	A3	-		7	A5	-	**6**	-	-
BLB	PI 275185	MEX	19.21N	99.12W	18	18	10	4	A1, M1	-		9	A2	-	**2**	-	-
BLB	PI 275197	MEX	19.21N	99.12W	7	7	1	1	A3	3	ND	2		-	-	-	-
BLB	CGN 17687	MEX	19.21N (b)	99.12W	10	10	9	6	L1,G1	-		10	A1	-	**6**	-	-
BLB	PI 498225	MEX	19.25N	100.20W	9	9	9	9	A1, M1	-		9	A2		**9**		
BLB	PI 347757	MEX	19.31N	100.15W	9	9	7	5	C3, F1	-		7	A2	-	**5**	-	-
BLB	CGN 17690	MEX	19.50N	101.43W	10	10	3	-	M1	-		7	A1	-	-	-	-
BLB	PI 545751	MEX	20.59N	103.10W	10	10	9	8	B3	-		8	A2		**8**		
BLB	PI 590930	MEX	21.01N	102.59W	7	7	7	6	J1, K1	-		7	A3		**6**		
BLB	PI 498224	MEX	19.24N	100.21W	10	10	10	9	B2	-		9	A2	-	**9**	-	-
BLB	CGN 18310	MEX	ND	ND	12	11	-	-	M1	-		5	B3	-	-	-	-
BLB	CGN 18311		ND	ND	7	7	-	-		-		7	B6	-	-	-	-
DPH	CGN 24119	MEX	18.55N	99.43W	10	10	10	10	C6	-		-		-	-	-	-
DPH	PI 253210	MEX	19.00N (b)	99.06W	5	-	-	-		-		5	A2	-	-	-	-
DPH	CGN 17688	MEX	19.42N	103.31W	15	15	8	8	A6	-		15	A2, A4, A6	-	**8**	-	-
PTT	CGN 21363	GTM	15.09N	90.18W	11	11	-	-	M1	-		11	B2, B8	-	-	-	-
PTT	PI 604065	GTM	15.10N	90.18W	9	9	-	-		2		7	B2	-	-	**2**	-
PTT	CGN 21364	GTM	15.13N	90.18W	11	11	-	-		-		11	B2	-	-	-	-
PTT	CGN 22732	GTM	15.19N	91.31W	11	10	-	-		-		7	B4, B5	-	-	-	-
PTT	CGN 23075	GTM	15.19N	91.33W	11	11	-	-	M1	-		11	B6	-	-	-	-
PTT	CGN 23074	GTM	15.46N	91.30W	12	10	-	-		-		12	A2, B1, B7	-	-	-	-

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S1b. *S. brachistotrichum*, *S. cardiophyllum*, *S. nayaritense* and polyploid *Solanum* accessions

Taxon	Accession number	Country	Coordinates		Number of genotypes	Rpi-blb genes identification								Rpi-blb genes Combination			
			lat	lon		RGA1	Blb1 F/R	Blb1 1/1´	Haplotype	Blb2 F/R	Haplotype	Blb3 F/R	Haplotype	blb1/ blb2	blb1/ blb3	blb2/ blb3	blb1/ blb2/ blb3
BST	GLKS 2717	MEX	ND	ND	6	1	1	-		-		3	A2	-	-	-	-
CPH	CGN 22387	MEX	ND	ND	12	10	5	3	D1	1	B	-		1	-	-	-
CPH	CGN 18326	MEX	ND	ND	12	10	4	4	D1	1	B	3	A1	-	1	-	-
FEN	CGN 22370	MEX	25.25N	101.00W	10	9	-	-		-		-		-	-	-	-
FEN	CGN 24120	MEX	28.08N	107.30W	10	9	-	-		-		-		-	-	-	-
FEN	CGN 23984	MEX	28.19N	107.21W	10	9	-	-		-		-		-	-	-	-
FEN	CGN 24121	MEX	29.08N	108.08W	10	9	-	-		-		-		-	-	-	-
FEN	CGN 17715	USA	31.26N	110.19W	10	9	-	-		-		-		-	-	-	-
FEN	CGN 18063	USA	31.54N	109.16W	10	9	-	-		-		-		-	-	-	-
FEN	CGN 18116	USA	33.23N	108.36W	11	9	-	-		-		-		-	-	-	-
HJT	CGN 18345	MEX	24.52N	100.13W	11	10	-	-		-		1	A1	-	-	-	-
HJT	CGN 17717	MEX	25.25N	100.51W	11	10	-	-		-		1	A1	-	-	-	-
HJT	CGN 17718	MEX	25.25N	101.00W	11	9	-	-		-		1	A1	-	-	-	-
HJT	CGN 22369	MEX	25.25N	100.51W	10	10	-	-		-		-		-	-	-	-
PLT	CGN 22361	MEX	20.20N	100.00W	10	10	-	-		-		-		-	-	-	-
PLT	CGN 22362	MEX	20.20N	100.00W	10	10	-	-		-		-		-	-	-	-
PLT	CGN 17751	MEX	22.47N	102.35W	5	5	-	-		-		-		-	-	-	-
PLT	CGN 17750	MEX	ND	ND	10	10	-	-		-		-		-	-	-	-
PLT	CGN 18318	MEX	ND	ND	10	10	-	-		-		-		-	-	-	-
PNT	CGN 17740	MEX	20.36N	100.23W	11	-	-	-		-		6	A1	-	-	-	-
PNT	CGN 17745	MEX	20.40N	103.20W	8	-	-	-		-		1	A1	-	-	-	-
PTA	CGN 18309	MEX	23.20N	105.07W	10	10	-	-		-		-		-	-	-	-
PTA	CGN 17831	MEX	23.30N	103.35W	11	10	10	10	C4, C5	-		-		-	-	-	-
PTA	CGN 18319	MEX	24.11N	105.02W	10	10	-	-		-		-		-	-	-	-
PTA	CGN 17832	MEX	ND	ND	10	10	3	3	C4, C6	-		-		-	-	-	-
PTA	CGN 17830	MEX	24.57N	103.54W	10	10	-	-		-		-		-	-	-	-
PTA	CGN 22385	MEX	ND	ND	10	10	-	-		-		-		-	-	-	-
STO	CGN 18333	MEX	19.05N	98.41W	12	11	-	-		-		2	A1	-	-	-	-
STO	CGN 23072	MEX	19.05N	98.41W	10	10	-	-		-		-		-	-	-	-
STO	CGN 23519	MEX	19.07N	98.46W	10	10	10	10	D1	-		-		-	-	-	-
STO	CGN 18348	MEX	19.35N	99.04W	11	10	-	-		-		1	A1	-	-	-	-
STO	CGN 17605	MEX	19.42N	101.07W	10	10	8	8	B3	-		-		-	-	-	-
STO	CGN 17607	MEX	19.42N	101.07W	10	10	-	-		-		-		-	-	-	-
STO	CGN 17606	MEX	ND	ND	10	10	-	-		-		-		-	-	-	-
STO	CGN 18332	MEX	ND	ND	10	10	-	-		-		-		-	-	-	-
STO	CGN 18334	MEX	19.15N (a)	98.53W	10	10	-	-		-		-		-	-	-	-

(a): Coordinates estimated with Google Earth using the CGN route description. (b): Data derived from Heijmans (2007). Abbreviations:ND: not determined BLB: *S. bulbocastanum*, BST: *S. brachistotrichum*, CPH: *S. cardiophyllum*, DPH: *S. bulbocastanum sbsp dolichophyllum*, FEN: *S. fendleri*, HJT: *S. hjertingii*, PLT: *S. polytrichon*, PNT: *S. pinnatissectum*, PTA: *S. papita*, PTT: *S. bulbocastanum* subsp. *partitum*, STO: *S. stoloniferum*.

The primer pair Blb2F/R (Wang et al. 2008) amplified *Rpi-blb2* in 7 accessions of *S. bulbocastanum* and in 2 *S. cardiophyllum* accessions from 6 different locations (Table S1 and Figure 1A).

The primers specific for *Rpi-blb3* amplified distinct fragments in 35 accessions from 28 different locations. Accessions showing amplification originated from 26 *S. bulbocastanum*, 2 *S. pinnatissectum,* 1 *S. brachistotrichum*, 1 *S. cardiophyllum*, 3 *S. hjertingii* and 2 *S. stoloniferum* accessions (Table S1 and Figure 1A).

The *Rpi-blb1* and *Rpi-blb3* gene combinations were found in genotypes from 14 *S. bulbocastanum* accessions originating from 12 different locations. The *Rpi-blb1* and *Rpi-blb2* gene were found together in genotypes from 5 *S. bulbocastanum* accessions from 2 different locations. The *Rpi-blb2* and *Rpi-blb3* fragments were found together in 2 *S. bulbocastanum* accessions from 2 distinct locations (Table S1 and Figure 1B).

The percentages of the individual genotypes of *S. bulbocastanum* containing *Rpi-blb1*, *Rpi-blb2* or *Rpi-blb3* fragments are 36%, 9% and 56%, respectively.

Three genotypes, belonging to PI498223 and PI545711 accessions, harbored all three genes. Only in a small number of accessions all genotypes harbored the same gene; 2 accessions were fixed for *Rpi-blb1* and 10 for *Rpi-blb3*. Interestingly 91% of the genotypes from the subspecies *S. bulbocastanum* subsp. *partitum* contained *Rpi-blb3*.

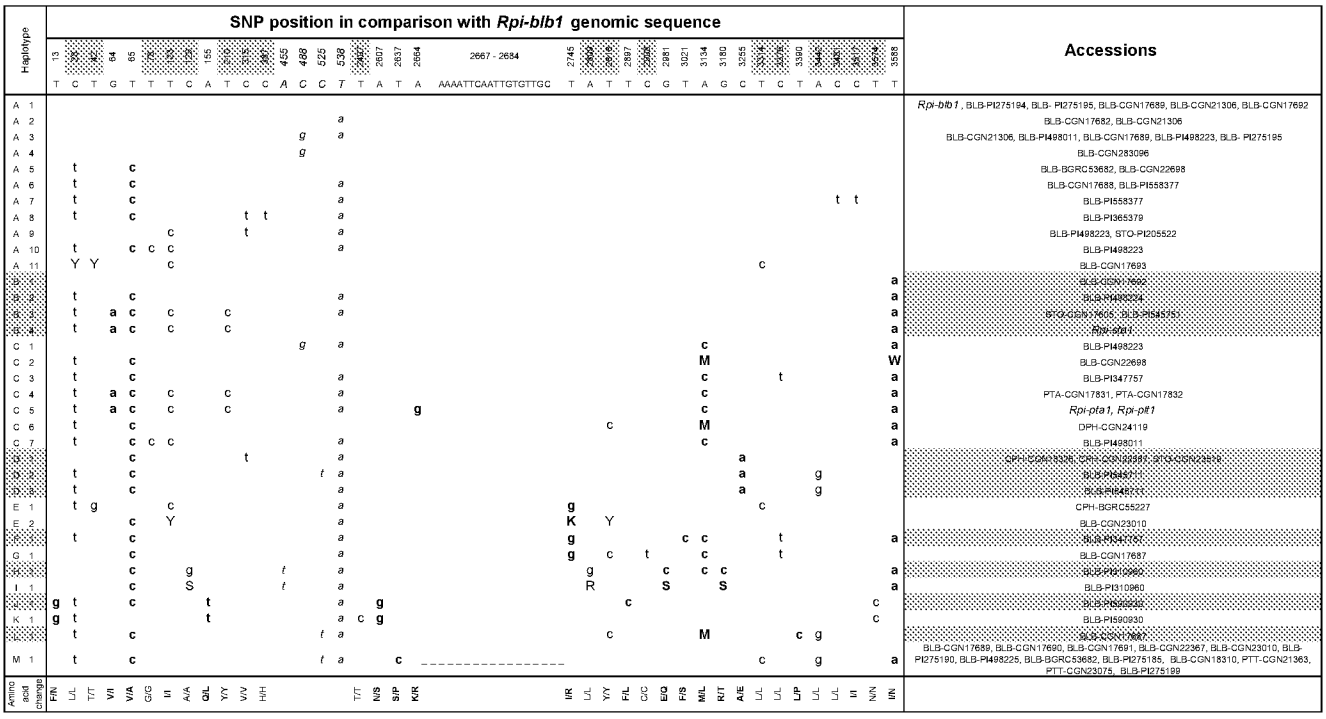
Allelic variation in the Rpi-blb1, Rpi-blb2 and Rpi-blb3 gene fragments

To verify the nature of the amplified fragments and to explore the R-gene diversity at the nucleotide level, part of the gene was sequenced for all the positives accessions. The results are presented in Table 1.

The *Rpi-blb1* amplified sequence spans 1734 base pairs in total, composed of two separate parts: the first part is from the start codon to position 545 obtained with primer pair 517/ 1519. The second part is from position 2404 to the stop codon obtained with primer pairs Blb1F/R, 1531/519 and 1/1'. Sequence alignment revealed a total of 56 SNPs compared to the cloned *Rpi-blb1* gene with 34 SNPs in the coding region, 4 SNPs in an intron part and the deletion of 18 nucleotides which is characteristic for the *Rpi-blb1* non-functional allele.

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Table 1a: Allelic variation for *Rpi-blb1* in *S. bulbocastanum* accessions and related species.



The *Rpi-blb1* sequence spans 1709 base pairs (bp) in total, composed of two separate parts: (1) from the start codon to position 520 derived from the primer 517/1519. In italic are the located in the intron sequence. (2) from position 2404 to stop codon derived from the primer BLB1F/R and 1521/519.

Table 1b: Allelic variation for *Rpi-blb2* in *S. bulbocastanum* accessions and related species.

Haplotype	SNP position in comparison with *Rpi-blb2* genomic sequence																									Accessions
	2226-2297	2298	2299	2300	2302	2303	2304	2305	2306	2308	2309	2310	2311	2312	2313	2315	2316	2317	2318	2319	2320	2321	2322	2323	2324	2325	2326	
A	TCTGGAAGAAGTGGTGAAAATTTATTTGGATGATTTAATTTCCAGTAGCTTGGTAATTTGTTTCAATGAGAT	A	G	G	G	A	T	T	A	C	C	T	A	C	T	G	C	C	A	A	C	T	T	C	A	T	G	PI 498011, BLB-P498233, BLB
B	'	C	A	C	T	T	G	G	G	T	T	A	A	T	C	A	G	A	T	T	T	T	G	T	A	G	T	CPH-CGN18326, CPH-CGN22387, CPH-BGRC55227

The *Rpi-blb2* sequence spans 715 bp derived from the primers Blb2F/R

Haplotypes code are indicated in the left column. Nucleotide positions are numbered according to the genomic sequences of the genes, beginning at the START codon and including introns in the case of *Rpi-blb1* and *Rpi-blb2*. Amino acid changes are indicated on the bottom line. SNP's part of the intron region are in italic. In light grey are highlighted the position where SNP's did not change the amino-acid. SNP's resulting in non-synonymous amino-acid change are indicated in bold. *Solanum* accessions in which the haplotypes where identified are indicated on the right column.

Nucleotides abbreviations according to the IUB code: K, GT; M,AC; R, AG; S, GC; W,AT; Y, CT.

Table 1c: Allelic variation for *Rpi-blb3* in *S. bulbocastanum* accessions and related species.

Haplotype	SNP position in comparison with *Rpi-blb3* genomic sequence	Accessions
	941 972 1001 1016 1021 1027 1026 1036 1043 1045 1065 1103 1107 1116 1131 1133 1196 1235 1246 1255 1258 1272 1293 1309 1326 1335 1352 1371 1396 1439 1487 1501 1510	
	G T G T G G G A G T A G A G A A T C G C T T G C G C G C C c C T G	
A 1		PNT-CGN17745, PNT-CGN17740, *Rpiblb3,* DPH-CGN17688, PTT-CGN23074, BLB-PI545711, BLB-CGN17690, BLB-CGN17687, HJT-CGN22370, HJT-CGN18345, PNT-CGN17742, STO-CGN18348, STO-CGN18333
A 2	a	BLB-CGN22698, BLB-CGN23010, BLB-PI 275185, BLB-BGRC53682, BLB-PI275190, BLB-PI310960, BLB-PI347757, BLB-PI498224, BLB-PI498225, BLB-PI545711, BLB-PI545751, BLB-CGN176888, DPH-PI253210, BST-GLKS32717, PTT-CGN23074, BLB-GLKS31741
A 3	c	BLB-PI590930
A 4	c	DPH-CGN17688
A 5	g	PTT-CGN17689
A 6	g *	DPH-CGN17688
A 7	* a	BLB-BGRC53682
A 8	t	PTT-PI275200
B 1	a t a	PTT-CGN23074
B 2	a a a t a	PTT-CGN21364, BLB-PI604065, PTT-CGN21363
B 3	a a a t a g	BLB-CGN18310
B 4	a a t a	PTT-CGN22732
B 5	a a a t a	PTT-CGN22732
B 6	c a a t a t	BLB-CGN18311, PTT-CGN23075
B 7	c a a t a	PTT-CGN23074
B 8	Y K R s R Y M	PTT-CGN21363
C 1	a a a t t	STO-PI205522
C 2	a t a t t	BST-CPC3822, BST-PI320265
C 3	a c t t t	BST-PI320265
C 4	a c t t t	NYR-PI545825
C 1	c c a	*R2-like, Rpi-abpt*
E 1	t a c t a	*R2*
Amino acid change	R/I S/S R/K V/A A/S V/I M/I M/L S/N L/L E/D K/K G/G S/N fs fs I/N T/S A/A I/L Y/H F/L K/K R/W E/E P/P R/I D/E R/stop T/S A/V F/L V/I	

The *Rpi-blb3* sequence spans 618 bp derived from the primers Blb3F/R

The 34 haplotypes could be classified into 13 groups (A-M) based on the identity of the non-synonymous SNP changes (Table 1a). Each haplotype group contained 1 to 18 accessions. Group A, which is the dominant group, contains haplotypes without non-synonymous SNPs and is only composed of *S. bulbocastanum* accessions and the cloned *Rpi-blb1* gene. Group B additionally contains *S. stoloniferum (Rpi-sto1* like) haplotypes while *S. papita* and *S. polytrichon* (*Rpi-pta1* and *Rpi-plt1* like) are part of group C. Haplotype C2, A11, C6, E2, I1 and L1 display double SNPs which are probably the result of heterozygous genotypes. Haplotype M1 represents the known non functional allele, identified by the 18 bp deletion (Song et al. 2003).

The *Rpi-blb2* amplified sequence spans 715 base pairs from position 2157 to 2872 derived from the primers Blb2F/R (Wang et al. 2008). From the 2 sequences obtained, one is 100% identical to the *Rpi-blb2* sequence and the second one displayed a variable region composed of 72 nucleotide deletion at the beginning of the sequence followed by 26 SNPs in the remaining part (Table 1b). The *Rpi-blb3* amplified sequence spans 618 base pairs from position 918 to 1536. Sequence alignment reveals 33 SNPs displayed in 22 haplotypes containing 1 to 16 accessions (Table 1c). Our classification differentiates 5 groups containing 1 to 34 accessions, according to the type of polymorphism present in the sequence. Group A displays one SNP per haplotype, A1 and A2 being the most commonly present in 12 and 16 accessions, respectively. Haplotype A6 and A7 show one nucleotide deletion leading to a frame shift. Group B haplotypes have 4 SNPs in common clearly defining a polymorphic sequence block. Haplotypes B are exclusively identified in accessions originating from Guatemala (Table S1 and Appendix 1). Haplotype B6 contains a SNP in position 1396 resulting in a stop codon. Group C comprises haplotypes with 4 SNPs in common defining as well a polymorphic sequence block, present in *S. brachistotrichum*, *S. nayaritense* (Table 2) and *S. stoloniferum*. The *R2-like*, *Rpi-abpt* and *R2* alleles are classified in haplotypes D1 and E1.

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Validation of Late Blight resistance by effector recognition and detached leaf assay

In an attempt to link the presence of *Rpi-blb* haplotypes to Late Blight resistance, a set of 50 plants from our in vitro collection was screened. It included a molecular characterization by PCR-amplification with *Rpi-blb1, Rpi-blb2* and *Rpi-blb3* gene specific primers followed by sequencing the PCR fragment. An additional combination of Rpi-blb3 primer, blb3 F/Ra was used, with Ra being specific to *Rpi-blb3* and its homolog *R2-like* (Lokossou et al. 2009), in order to pick up intermediates of *Rpi-blb3* and *R2-like.*

Phenotypic characterization was carried out through detached leaf assays with six *P. infestans* isolates (Table S2) and agro-infiltration with Pi-effectors interacting with *Rpi-blb1* and *Rpi-blb3*. The chosen *P. infestans* isolates were genetically very different from each other, as it has been shown by simple sequence repeat (SSR) fingerprinting (Champouret et al. 2009), and display different virulence spectra with respect to the *Rpi-blb* genes tested. The *Rpi-blb2* gene confers resistance to the six isolates used and the *Rpi-blb1* and *Rpi-blb3* gene confer the resistance spectrum shown in Table 2. None of the tested plants contained *Rpi-blb2*, as no fragment was amplified with the primers Blb2F/R. Unfortunately, we observed background responses caused by the agro-infiltration of Avrblb2 in all the plants tested (not shown), thus *Rpi-blb2* presence or absence could not be verified by effector recognition in wild *Solanum* species. Results obtained for *Rpi-blb1* and *Rpi-blb3* are presented in Table 2 and show the diversity of possible interactions, classified in 5 categories.

Table 2: Evaluation of *Solanum* genotypes by molecular characterization, effector agro-infection and detached leaf assay. Form the left to the right are indicated in the column the *Solanum* accession taxon; identification number (genotype number in bracket); geographic coordinates (latitude-longitude); results of the specific amplification of *Rpi-blb1* by the primers combination 1/1' and *Rpi-blb3* by blb3F/R and blb3 F/Ra with Ra being specific to *Rpi-blb3* and *R2-like* (Lokossou et al. 2009); the haplotype identified after sequencing of the specific fragment referring to table 1; results of the effector agro-infection with Avr-blb1, Pi-Avr2 and the controls R3a and Pi-Avr3a/R3a together, are indicated by degree of response intensity from (-) meaning no response to (+++) meaning clear hypersentive reaction; results of the detached leaf assay conducted with the isolates PIC99177, PIC 99189, PIC 99183, IPO-C, 90128 and UK7824 scored R (resistant), S (susceptible), Rq (quantitatively resistant), Q (quantitative).

Abreviations: ND: not determined sequences, BLB: *S. bulbocastanum*, BST: *S. brachistotrichum*, CPH: *S. cardiophyllum*, DPH: *S. bulbocastanum* subsp. *dolichophyllum*, FEN: *S. fendleri*, HJT: *S. hjertingii*, NYR: *S. nayaritense*, PLT: *S. polytrichon*, PNT: *S. pinnatisectum*, PTA: *S. papita*, PTT: *S. bulbocastanum* subsp. *partitum*, STO: *S. stoloniferum*. CPH-BGRC55227* contains *Rpi-blb2* haplotype B.

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Taxon	Accession number	Coordinates		Specific amplification			Sequence		Effector Agro-infiltration				*Phytophtora infestans Detached leaf assay*						Comments
		lat	lon	Rpi-blb1	Rpiblb3	Rpiblb3	Rpiblb1	Rpiblb3	Avr-blb1	Pi-Avr2	Controls		PIC99177	PIC99189	PIC99183	IPO-C	90128	UK7824	
				1/1'	F/R	F/Ra	Haplotype				R3a	R3a/Avr3a							
BLB	CGN17692 (Blb8005-8)	17.53N	96.33W	x		x	A1		+ + +	-	-	+++	S	S	R	R	R	R	Genotype containing Rpi-blb1
DPH	CGN17688 (Blb99-256-3)	19.42N	103.31W		x	x		A1	-	+ + +	-	+++	Q	S	R	S	R	S	Genotype containing Rpi-blb3
HJT	CGN17718	25.25N	100.00W			x		A1	-	+ + +	-	+++	S	S	Rq	S	R	Q	
HJT	CGN22370	25.25N	101.00W		x			A1	-	+ + +	-	+++	S	S	R	S	R	S	
HJT	CGN18345	24.52N	100.13W		x			A1	-	+ + +	-	+++	Q	S	R	S	R	S	
HJT	BGRC32671	ND	ND		x			A1	-	+ + +	-	+++	S	R	Rq	S	R	S	
HJT	CGN17717	25.25N	100.51W		x	x		A1	-	+ + +	-	+++	R	S	R	S	R	S	
STO	CGN18333	19.05N	98.41W		x			A1	-	+ + +	-	+++	S	R	R	S	R	S	
PNT	CGN18331	ND	ND		x	x		A1	+	+	-	+++	R	R	R	R	R	R	Plants with Avr2 recognition and Rpi-blb3 fragment.
BST	CGN17681	ND	ND		x	x		A2	-	+ + +	-	+++	S	S	R	S	Rq	S	
BLB	CGN18310	ND	ND		x			B2	-	+ +	-	+++	R	R	R	Q	R	R	
PTT	CGN21364	15.13N	90.18W		x			B2	-	+	-	+++	R	Q	R	R	R	S	
BST	PI320265	29.08N	106.05W			x		C2	-	+ + +	-	++	S	R	R	S	R	Q	
BST	PI320265	29.08N	106.05W			x		C3	-	+ + +	-	+	S	S	R	S	R	S	
NYR	PI545825	ND	ND		x			C4	-	+ + +	-	+++	Q	R	R	S	R	R	
STO	PI205522	19.07N	98.46W	x	x		A9	C1	+ + +	+ + +	-	+++	Q	R	R	R	R	R	Plant with Avr2 and AvrBlb1 recognition and Rpi-blb1 and Rpi-blb3 fragment.
BLB	CGN17689	19.21N	99.12W	x	x		A 1	A5	+ + +	-	-	+++	R	R	R	R	R	R	
BLB	CGN21306	17.30N	96.27W	x	x		A1	nd	+ + +	-	-	+++	R	R	R	R	R	R	Plants with AvrBlb1 recognition and Rpi-blb1 fragment.
BLB	CGN17692	17.53N	96.33W	x		x	A1		+ + +	-	-	++	R	S	R	R	R	R	
STO	CGN18348	19.35N	99.04W		x			A1	+ + +	-	-	+	R	S	R	R	R	R	Plants with AvrBlb1 recognition and no Rpi-blb1 fragment.
BLB	CGN17693	19.43N	99.47W						+	-	-	++	R	R	R	R	R	R	
STO	CGN18333	19.05N	98.41W		x			A1	-	-	-	++	Q	R	S	S	R	R	Plants with Rpi-blb1 and/or Rpi-blb3 fragment
BLB	BGRC53682	19.15N	98.53W	x	x	x	A5	A2	-	-	-	+++	R	S	R	R	R	R	
PTT	PI275200	15.10N	91.31W		x			A8	-	-	-	+++	Q	Q	Q	S	Q	R	
PTT	CGN23075	15.19N	91.33W		x			B6	-	-	-	+++	R	R	R	R	R	R	
BLB	CGN22698	19.11N	98.13W	x		x	A5		-	-	-	+++	R	R	R	R	R	R	
PNT	GLKS1586	ND	ND			x			-	-	-	+++	R	R	R	R	R	R	
CPH	GLKS99	ND	ND			x				-	-	+++	Q	S	S	S	S	R	
BLB	CGN18310	ND	ND			x			-	-	-	+++	Q	Q	S	S	S	Q	
PTA	CGN17830	24.57N	103.54W			x			-	-	-	+++	R	Q	S	S	S	S	
BLB	CGN23010	ND	ND						-	-	-	+++	R	R	R	R	R	R	
BLB	PI275199	19.29N	98.54W			x			-	-	-	+	R	R	R	R	R	R	
CPH	CGN22387	ND	ND						-	-	-	+	R	R	R	R	R	R	
CPH	CGN18325	ND	ND						+	-	-	+++	R	R	R	R	R	R	Plants containing different late blight resistance genes other than the tested Rpi-blb genes.
CPH	CGN18326	ND	ND						-	-	-	+++	Q	Q	S	R	Q	R	
CPH	CGN18326	ND	ND						-	-	-	+++	Q	R	Q	R	S	R	
CPH	BGRC55227	ND	ND						-	-	-	+++	Q	Q	S	R	S	R	
PTT	CGN23074	15.46N	91.30W		x			B1	-	-	-	++	S	S	S	S	S	S	
PTT	CGN21363	15.09N	90.18W		x			B2	-	-	-	+++	S	S	S	S	S	S	
PTT	CGN23074	15.46N	91.30W		x			B7	-	-	-	+	S	S	S	S	S	S	
BST	PI320265	29.08N	106.05W			x		C2	-	-	-	++	S	S	S	S	S	S	Late Blight suceptible plants
BST	CGN17681	ND	ND						+	-	-	+++	S	S	S	S	S	Q	
FEN	CGN18116	33.23N	108.36W						-	-	-	+++	S	S	S	S	S	S	
BST	CPC3822	ND	ND		x	x		nd	-	+ + +	-	+++	S	S	S	S	S	S	
STO	GLKS0620	ND	ND		x			nd	-	+	-	++	S	S	S	S	S	S	
PNT	CGN17743	ND	ND			x			+	++	++	++	R	R	R	R	R	R	
BLB	CGN22367	19.13N	98.48W			x			-	-	-	-	R	Q	R	R	R	R	
PNT	CGN17740	20.36N	100.23W		x			A1	+	+	++	++	R	R	R	R	R	R	Plants not suitable for agro-infiltration
PNT	CGN17742	21.07N	101.40W		x			A1	-	-	-	-	R	R	R	R	R	R	
BST	GLKS2717	ND	ND						-	+	+	+++	S	S	S	S	Q	S	

The first category comprises plants harboring the *Rpi-blb1* and/or *Rpi-blb3* gene, as judged from the marker analysis. They also interacted with the Avrblb1 and/or Pi-Avr2 elicitor and displayed a resistance spectrum identical or broader than the *S. bulbocastanum* derived *R*-genes. Based on the assay with Avrblb1, 3 *S. bulbocastanum* and 1 *S. stoloniferum* plants contained *Rpi-blb1*. PCR amplification and sequencing strengthened the presence of functional *Rpi-blb1* haplotypes, identified as A1 and A9.Their resistance spectrum matches with that of *S. bulbocastanum* plant (CGN17692), which was confirmed to harbor *Rpi-blb1* (van der Vossen et al. 2003). However, they were also resistant to isolates PIC99189 and PIC99177, known to break *Rpi-blb1* (Champouret et al. 2009), which suggests the presence of additional resistance factors in these genotypes. The *Rpi-blb3* haplotypes A1, B2, C1, C2, C3 and C4 were found in 14 plants from *S. bulbocastanum*, *S. brachistotrichum*, *S. hjertingii*, *S. nayaritense* and *S. stoloniferum* accessions. They all interact with Pi-Avr2 and display an identical or broader resistance spectrum in comparison to the genitor used to isolate *Rpi-blb3,* thus suggesting the presence of a functional *Rpi-blb3* haplotype and additional Late Blight resistance factors.

From plants classified in the second category fragments were amplified with primers specific for the *Rpi-blb1* and/or *Rpi-blb3* gene, but no interaction with Avrblb1 or Pi-Avr2 was observed. The *Rpi-blb* genes amplified might contain mutations preventing the interaction with the tested effectors. Moreover, these plants putatively contain Late Blight resistance genes other than the one tested, as judged from the responses to the isolates (Table 2).

The third category comprises resistant plants lacking *Rpi-blb1* or *Rpi-blb3* as judged from the marker assays and effector interaction. Interestingly, 2 *S. bulbocastanum* and 2 *S. cardiophylum* plants featured complete resistance to the full set of isolates. These plants probably contain late blight resistance genes other than the tested *Rpi-blb* genes. One *S. cardiophyllum* plant (CPH-BGRC55227*) was tested positive for *Rpi-blb2* haplotype B and displays a resistance spectrum not identical to the one of *Rpi-Blb2* containing plant.

Late Blight susceptible plants not containing any functional *R*-gene as judged from the response with the tested isolates, but showing amplification with *Rpi-blb3* primers, are found in category 4. One *S. brachistotrichum* clone contains *Rpi-blb3* haplotype C2 and interacts strongly with Pi-Avr2 but is completely susceptible to the set of *P. infestans* isolates tested. Three accessions from *S. bulbocastanum* subsp. *partitum*, 2 of *S. brachistotrichum* and 1 of *S. stoloniferum* contain *Rpi-blb3* haplotypes B1, B2, B7 and C2 but do not interact with Pi-Avr2 and display a susceptible phenotype when infected with the set of isolates.

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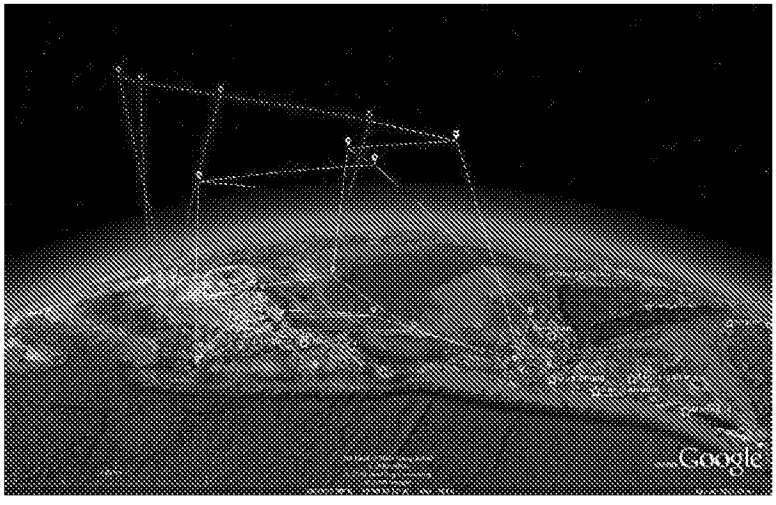
The last category of plants was not suitable for the agro-infiltration. The reactions with the effectors were non specific and resulted in a hypersensitive response to all the tested effectors, or gave no reaction to control *Pi-Avr*/*R*-gene co-infiltration (Table 2). However plants showing resistance to all tested isolates are likely to contain new late blight resistance gene.

Geographical distribution of the Rpi-blb genes

The 3 *Rpi-blb* genes are mainly present in the *S. bulbocastanum* accessions from Central Mexico (Figure 2) and plants not containing the 3 studied *Rpi-blb* genes originate from the northern part of Mexico and the southern part of the USA. Also the allotetraploid species *S. fendleri*, which is found in the north of Mexico, lacks the *Rpi-blb* genes tested.

Solanum stoloniferum from the Durango region contains *Rpi-blb1* while *Rpi-blb3* is present in *S. hjertingii* originating from Monterray, *S. brachistotrichum* from Chihuahua and *S.bulbocastanum* subsp. *partitum* from Guatemala.

In order to see if haplotypes diverge when they originate from more geographically distant areas, the haplotypes were phylogenetically mapped in Google Earth using Supramap (Janies et al. 2007). From the results, it can be seen that the M1 haplotype of *Rpi-blb1* (Appendix 1) is present in Central Mexico as well as in Guatemala and that the less supported clade containing the C and B haplotypes from *S. stoloniferum* is confined to the upper part of Mexico. For *Rpi-blb3*, the haplotypes of group C originate from Central towards Northern Mexico, while haplotypes recognized as group B all are from Guatemala.



Appendix 1 (KMZ file): *Rpi-blb* genes diversity and geographical origin. Phylogenetic and geographical localization of genotypes containing *Rpi-blb* haplotypes. *Rpi-blb3* groups B and C haplotypes and *Rpi-blb1* haplotype M1 are indicated in red, blue and white, respectively. The original kmz file can be provided as and be opened in a Google Earth desktop application. Additionally to the phylogenetic tree, the location of the collection sites of the accessions can be visualized.

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A.

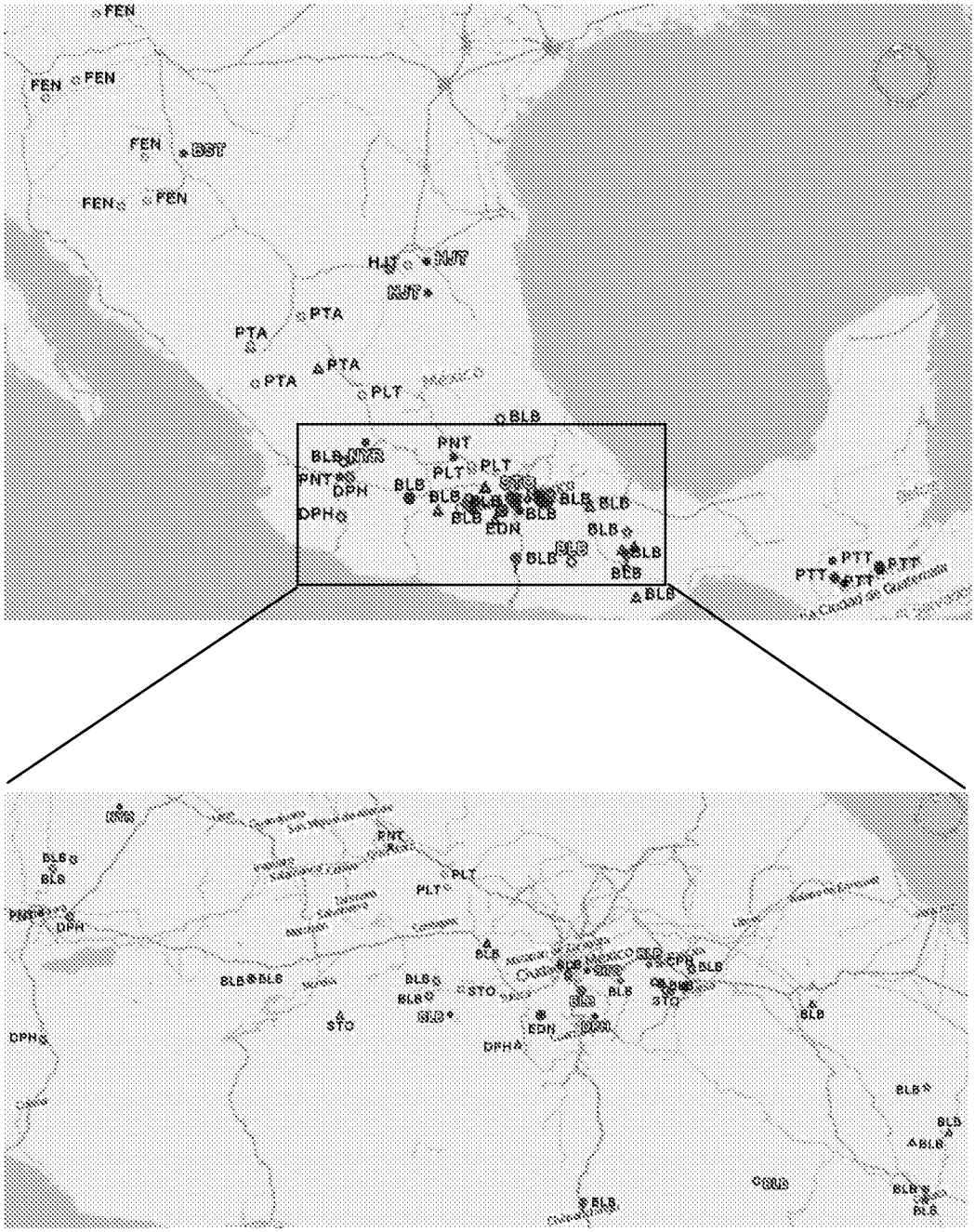


Figure 2: Geographical location of the *Solanum* accessions under study containing only *Rpi-blb1* (blue triangular), only *Rpi-blb3* (red dots), *Rpi-blb1* and *Rpi-blb3* (purple lozenges), *Rpi-blb1* and *Rpi-blb2* (green stars), the three *Rpi-blb* genes (red drops) and the accessions not containing any Rpi-blb gene (yellow dots)

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DISCUSSION

The use of gene specific primers and gene functionality

Gene specific primers are powerful tools for the evaluation of presence or absence of resistance genes (Arens et al. 2010). Although *R*-genes often have numerous close homologues, in most cases it is possible to develop markers in such a way that a fragment specific to the gene of interest is amplified. Subsequent sequencing of the fragment makes it possible to verify its identity, to differentiate the haplotypes and to discard recognized pseudo-genes thus indicating the presence or absence of most likely functional genes. We validated this approach by phenotyping 50 individual plants using effectors and detached leaf assays. The plants were first characterized for the presence of *Rpi-blb1, Rpi-blb2* and *Rpi-blb3* with markers. In the 50 cases we only observed two cases for *Rpi-blb1* in which the markers predicted the presence of the gene where there was no effector response. This indicates that there is a good but not 100% correlation between markers and phenotype for *Rpi-blb1* (table 2). All plants tested negative when using the gene specific markers for *Rpi-blb2*. Unfortunately, this could not be validated by an effector response due to background that was observed in the agro-infiltration experiment with Avrblb2. For *Rpi-blb3* we found 13 cases in which there was amplification with the gene specific primers but no response to the effector. Also there were 3 cases in which there was a response to the effector but no amplification of the gene specific fragment and one case in which we observed an effector response and no resistance in the DLA assay. An additional primer pair (Rpiblb3 F/Ra) did amplify in some of the cases where there was no amplification with the *Rpi-Blb3* specific primers was found (Table 2), suggesting that similar but slightly different genes are present. This was also clear from the sequence analysis of some of the fragments obtained with the *Rpi-blb3* specific primers (e.g. the B and C haplotypes of *Rpi-blb3*). These results reflect the complexity of *Rpi-blb3* gene family and underline the usefulness of the combined techniques to identify *Rpi-blb3* from the other alleles of the cluster. However, it should be stressed that in most cases where there was a discrepancy observed between marker assays and phenotypic assays this was outside *S. bulbocastanum*.

Discrepancies between molecular and functional assays can have several causes. This can be the case when plants containing an *R*-allele specific fragment are for some reason unable to interact with the Avr-protein. In such cases the *R*-gene fragment found is probably not translated into a protein interacting with the Avr-protein. Hall et al. (2009) studied natural variation of *Atr13* in a set of *Hyaloperonospora arabidopsidis* isolates and showed differential interaction of *Atr13* with *Rpp13* alleles. The *Atr13* alleles are recognized by *Rpp13* alleles and by *R* genes or genes at other loci in *A.*

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thaliana. Interestingly, the *Rpp13-Rld* allele is unable to recognize *Atr13* alleles, and most probably detects an alternative effector protein in *H. arabidopsidis* isolates. Recognition specificity of different effectors by different alleles has previously also been demonstrated at the *RPM1* locus of *A. thaliana* (Bisgrove et al. 1994; Grant et al. 1995), at the *L* locus in flax (Dodds et al. 2004) and at the *Pto* locus in tomato (Kim et al. 2002; Ronald et al. 1992).

Also, plants may produce an *R*-allele fragment and show recognition of the Avr-protein (in our case for example in CPC3822), but still be susceptible to some of the key isolates. This might occur because of the high concentrations of the AVR-protein in our transient expression assay. A similar case was demonstrated by Schornack et al. (2004) for the Bs4/AvrBs3 interaction. Another explanation could be that the *R*-gene is probably active but unable to initiate a hypersensitive response in this specific phenotypic context. Finally, also plants not containing an *R*-allele specific fragment, but able to cause necrosis in presence of the Avr- protein will give discrepancies. Genotypes without any of these discrepancies and no indication for known *R*-gene presence, or plants with indication for known *R*-genes but with enhanced resistance spectra are good candidates to contain new *R*-genes.

Distribution of Rpi-blb genes in Solanum

The presence of *Rpi-blb1* and *Rpi-blb3* has been evaluated in approximately 1000 *Solanum* accessions. In addition, 72 accessions from *S. bulbocastanum* and related species were tested for *Rpi-blb1, Rpi-blb2* and *Rpi-blb3*. In total 35 *Rpi-blb1* haplotypes were found in the Mexican diploid species *S. bulbocastanum* and *S. cardiophyllum* as well as in the polyploid Longipedicellata *S. stoloniferum* (including *S. papita* and *S. polytrichon*), all geographically located in Central and South Mexico. Although the data have to be treated with caution, similar observations were made for *Rpi-blb3* were 22 haplotypes were found in the Mexican diploids *S. bulbocastanum*, *S. cardiophyllum S. pinnatissectum, S. nayaritense*, and *S. brachistotrichum* , as well as in the polyploid Longipedicellata *S. stoloniferum* and *S. hjertingii*. Interestingly, one *Rpi-blb3* haplotype was exclusively found in *S. bulbocastanum* originating from Guatemala thus showing a clear geographical/ *R*-gene haplotype association. The *Rpi-blb2* gene was only identified in a small number of *S. bulbocastanum* accessions from the Mexican provinces Oaxaca, Guerrero and Toluca. Since no haplotype variation within *Rpi-blb2* was found, this is a strong indication that this *R*-gene is from recent origin, as was suggested by Wang et al. (2008)

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The results for the 3 genes show that their spread is limited to the diploid Mexican and Longipedicellata group and confined to Central America. The occurrence of similar haplotypes in different diploid species may be explained by the fact that these species are able to form interspecific hybrids, especially *S. bulbocastanum* and *S. cardiophyllum* (Graham 1965). The presence of both *Rpi-blb1* and *Rpi-blb3* in the polyploid Longipedicellata may be explained by the fact that this group of *Solanum* section *Petota* is of allotetraploid origin. It has been shown that *S. hjertingii*, for example, consists of an *S. verrucosum* like and an *S. cardiophyllum* like genome (Pendinen et al. 2008). Wang et al. (2008) suggested that *S. bulbocastanum* may be one of the parental species of the allotetraploid *S. stoloniferum*. The observation that the Late Blight resistance genes evaluated are confined to Central America might be true for the distribution of other *R*-genes as well.

Rpi-blb genes are found in different frequencies in S. bulbocastanum populations

Genebank accessions used in this study consist of plant material that has been generatively multiplied by the genebank prior to usage. In the Netherlands, at CGN, this is done without any *P. infestans* pressure, since protective spraying is applied every week. This might have resulted in slight frequency changes in the presence of the *Rpi-blb* genes compared to the actual situation in Mexico.

We assessed the frequency in which the different genes are present in the *S. bulbocastanum* accessions under study. Individual plants containing all 3 *Rpi-blb* genes have been found at very low frequency (less than 1 %) and in two *S. bulbocastanum* accessions only, probably because of the rarity of *Rpi-blb2*. The *Rpi-blb1* and *Rpi-blb3* genes have been detected together in 27% of the *S. bulbocastanum* plants analyzed (52% of the accessions). Only *S. bulbocastanum* PI498225 is fixed for *Rpi-blb1* and *Rpi-blb3*, suggesting that accumulation of these genes in *S. bulbocastanum* is a rare event.

Pyramiding of resistance genes is suggested to enhance durable resistance under agricultural conditions (Park et al. 2009; Haverkort et al. 2009; Pink 2002) and is also observed in polyploid *Petota* species, like *S. demissum*. Although pyramiding of resistance genes appears to be a common mechanism in polyploids, in diploid species it seems to be rare with respect to *Rpi-blb1, Rpi-blb2* and *Rpi-blb3*. On the other hand, our functional test with the 50 plants showed frequently a broader resistance spectrum than expected with the tested *Rpi-blb* genes, indicating the presence of other, unknown, *Rpi*-genes.

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Almost all the accessions tested contain putative resistant and susceptible individuals. This might reflect a strongly fluctuating *P. infestans* pressure. The coexistence of resistant and susceptible individuals might also have an ecological meaning; in susceptible genotypes, newly recombined *R*-genes are under full pathogen pressure and functional *R*-genes are allowed to enter the population level.

The susceptible haplotype of *Rpi-blb1,* denoted as M1, is abundantly present and not confined to one specific geographical region, but scattered around in Central America (Appendix 1). The wide distribution of such a non-functional allele could imply that carrying a functional copy of *Rpi-blb1* affects the plants fitness in the absence of *P. infestans*. It is known that *R*-genes can result in serious fitness costs (Tian et al. 2003; McDowell and Simon 2006) and Kramer et al. (2009) showed that the *Rpi-blb1* gene is highly upregulated (37 times) once inoculated with *P. infestans*. Such observations suggest that we deal with a 'net cost of fitness under attack' (Korves and Bergelson 2004). In such cases null alleles might counterbalance potent but costly R-genes (McDowell and Simon 2006). Long-lived co-existence of resistant and susceptible alleles across the range of *Arabidopsis thaliana* ecotypes might be an example, as both alleles frequently occurred together within natural populations (Stahl et al. 1999). Another example would be RPS5 from *A. thaliana* (Henk et al. 1999).

Evolution of Rpi-blb genes

Sequence analysis of the fragments obtained for the *Rpi-blb1* and *Rpi-blb3* genes suggests an evolution through recombination and point mutations. In this respect there are clear differences between the 2 genes. The *Rpi-blb1* is thought to have resulted from a recombination event involving *RGA1-blb* and *RGA3-blb* (van der Vossen et al. 2003) and sequence analysis (Table 1) suggests a further evolution through point mutations, an evolutionary pattern typical for a Type II resistance gene as described for *R*-genes in natural lettuce populations (Kuang et al. 2004).

The situation with *Rpi-blb3* is more complex than with *Rpi-blb1* and most likely related to the complex nature of the cluster in which the gene is present. In this cluster several genes have almost identical sequences (Lokossou et al. 2009). Although all sequence information available has been taken into account for the design of the gene specific primers for *Rpi-blb3* it can not be excluded that some variants may not be discriminated by these primers. In *Solanum* as a whole even more *Rpi* genes have been mapped to the *Rpi-blb3* cluster. Examples are *Rpi-demf1* from *S. demissum* (Hein et al. 2007), *Rpi-Skn1* (Jacobs et al. 2010) and QTL's from *S. microdontum* (Colon et al. 1995; Micheletto et al.

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2000; Sandbrink et al. 2000). Such resistances might represent *R* genes which are homologues or recombinants sharing ancestry with *Rpi-blb3* as well as *R*-genes that have evolved independently and interact with other effectors.

The presence of conserved SNP's patterns among the haplotypes suggests recombinations between ancestors of *Rpi-blb3.* Such recombinations might have resulted in the *Rpi-blb3* group of B and C haplotypes which are similar to the *R2* gene as suggested by Lokossou et al. (2009).

Recent studies show that the recombination level at *R*-clusters is increased following pathogen infection, suggesting a mechanism that induces temporary genome instability in response to extreme stress, as recently reviewed by Friedman (2007). This instability might well be connected with the high intensity of transcription of some *R*-genes seen just after infection (e.g. Kramer et al. 2009) in which the genome temporary must be accessible for efficient transcription. It is conceivable that, depending on the disease pressure, we have a fast pattern of evolution of *R*-gene clusters dominated by sequence exchange or a slow pattern of evolution dominated by point mutations. Both evolutionary mechanisms appear to have shaped the *Rpi-blb1* and *Rpi-blb3* clusters. Additionally, the distinction between type I and II seems rather artificial; in real life a continuum is seen with type I and II as extreme borders.

The absence of mutation or recombination in *Rpi-blb2* fragment amplified from *S. bulbocastanum* and the low percentage of *Rpi-blb2* containing plants show that this gene emerged recently and didn't evolve yet.

New P. infestans R-genes identified

In our analysis, 76% of Late Blight resistant genotypes can not be explained by only the presence of the *Rpi-blb1, Rpi-blb2* and *Rpi-blb3* genes. Clear examples are *S. bulbocastanum* accession CGN23010 and P1275199, but also genotypes from CGN17689 and CGN21306 contain additional resistance gene(s), because of their resistance spectrum different from *Rpi-blb1*, *Rpi-blb2* or *Rpi-blb3* and thus providing new source(s) for future Late Blight resistance breeding. The *S. stoloniferum* accession PI205522, containing *Rpi-blb1* and *Rpi-blb3* based on the marker assays, contains an additional gene because of the resistance spectrum observed (Table 2). The gene involved is probably a paralog of $R4^{MA}$ (Van Poppel et al. 2009) as it confers resistance to isolate PIC99189 (avirulent on $R4^{MA}$, see supplementary Table 2) and a hypersensitive response with Avr4 effector (van Poppel et al. 2008) was found (unpublished results). It can not be excluded that the recently described gene *Rpi-bt1* (Oosumi et al. 2009), is responsible for some of the resistant phenotypes found in *S. bulbocastanum.*

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Since we found *Rpi-blb1* and *Rpi-blb3* in species related to *S. bulbocastanum* it would make sense that some of newly identified resistance genes in *S. bulbocastanum* might already have been identified in related species, like the resistances found in *S. cardiophyllum* and *S. pinnatisectum* (Kuhl et al. 2001).

CONCLUSION

Our study provides a clear picture of the distribution of Late Blight resistance genes so far isolated from *S. bulbocastanum.* It shows that *Rpi-blb1, Rpi-blb2* and *Rpi-blb3* have a very limited distribution among species. Few other species outside *S. bulbocastanum* contain the genes underlining the restricted interaction between *S. bulbocastanum* and other species. Several different haplotypes were detected for *Rpi-blb1* and *Rpi-blb3* and for 27 % of them functionality was shown. Our functional screen reveals the presence of more Late Blight resistance genes in *S. bulbocastanum*, confirming that this species is a rich source of Late Blight resistance genes as indicated by Budin (2002).

Allele mining in *Petota* germplasm with primers identifying known *R*-genes, allows the breeders to identify more amenable species for breeding. For example, *S. bulbocastanum* hardly crosses with cultivated potato (Helgeson et al. 1998), and the identification of *Rpi-blb1* alleles in *S. stoloniferum* largely facilitates the introgression breeding of *Rpi-blb1* for commercial usage (Vleeshouwers et al. 2008; Wang et al. 2008).

As observed by us and others (e.g. Budin. (2002)) a hotspot for resistance genes is found were host and pathogen co-evolve. This is also reflected by the putative new *R*-genes identified in this study. Given the high diversity of genes found, some promising *R*-genes might even not be present in the germplasm collections. New extensive collections in hot-spots of co-evolution and subsequent evaluations will undoubtedly provide new sources of resistance that can be used in breeding.

MATERIAL & METHODS

Plant material

The *Solanum* species used are listed in Table S1. They were obtained from the Centre for Genetic Resources of the Netherlands (CGN). DNA samples of 14 *S. bulbocastanum* accessions originating from the United States potato Genebank, in Sturgeon Bay, WI, USA, were kindly supplied

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by Dr. A. del Rio, Univ. Wisconsin, USA. (referred to as PI accessions in Table 1). When available up to 10 genotypes per accession were used. Individual clones listed in Table 2 were retrieved from the plant material described in Jacobs et al. (2008) for *in vivo* experiments consisting of detached leaf assays and effector agro-infiltrations.

PCR amplification, DNA sequencing and computer analyses

Specific primers used to amplify fragments of the 3 *R* genes studied are listed in Table S3 and Fig.S1. PCR products of randomly selected positive genotypes within an accession were sequenced directly to confirm their identity and to identify single nucleotide polymorphism (SNPs). Sequencing reactions were performed using a dye terminator cycle sequencing reaction kit (Perkin-Elmer, Pt Biosystem, Warrington, UK) and analyzed on an ABI3700 automated sequencer (Applied Biosystem, La Jolla, CA, USA). Multiple sequence alignments were conducted using DNASTAR Lasergen MegAlign version 8.0.2, permitting the distinction of the SNP's in comparison to the cloned *Rpi-blb* gene sequence (*Rpi-blb1* GenBank accession number AY426259.1; *Rpi-blb2* GenBank: DQ122125.1; *Rpi-blb3* GenBank: FJ536326.1). Unique sequences are reported in Table 1a and 1b. Nucleotides are numbered from the first nucleotide of the coding sequence of the cloned gene. The synonymous or silent SNP's not leading to a change in amino acid were differentiated from the non synonymous SNP's.

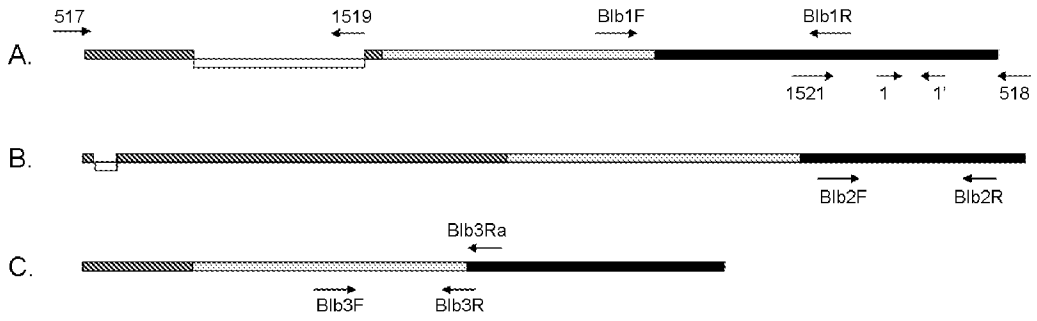


Figure S1: Location of *Rpi-blb* genes specific primers. *Rpi-blb1* (A), *Rpi-blb2* (B) and *Rpi-blb3* (C) nucleotide sequences are from the start to the stop codon and drawn to scale. The regions corresponding to the leucine zipper or the coil coiled, the intron, the NBS domain, and the LRR domain are highlighted in grey, white, light grey and black, respectively. Primer pairs used in this study are 517/1519, Blb1 F/Blb1 R, 1521/518, 1/1', Blb2F/Blb2R, Blb3F/Blb3R and Blb3F/Blb3Ra.

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Resistance assay

Detached leaf assays were used to determine the resistance phenotypes of the tested genotypes with 8 *Phytophthora infestans* isolates, PIC99177, PIC99189, PIC99183, IPO-C, 90128 and UK7824. Inoculum preparations and inoculations were performed as described by Vleeshouwers et al. (1999). Six days after inoculation, infection symptoms were scored on a scale from 1-8, to discriminate between very susceptible until very resistant; score 1-3 means clear infection with 1 heavily sporulating lesion and 3 ample sporulating lesion thus characterized as susceptible (S). Scores 4 and 5 are not clearly susceptible or resistant, therefore called quantitative (Q). Resistant (R) phenotypes are characterized by the score 6 with hypersensitive lesions larger than 10 mm, score 7 in between 4 and 10 mm and score 8 implicates hypersensitive lesions smaller than 4 mm in diameter.

Effector agro-infiltration on wild species

The Avr-effectors *Avrblb1* (Vleeshouwers et al. 2008) and *Avrblb2* were cloned into vector pK7WG2 and *Pi-Avr2* was cloned into vector pGRAB (Lokossou et al. 2009). The resistance genes *R3a* (Huang et al. 2004) and its cognate effector *Pi-Avr3a* (Bos et al. 2006) were cloned into the vector pGRAB (kindly provided by S.N. Chapman) and used as control for the agro-transformation efficiency. The constructs were transferred into the same *A. tumefaciens* strain AGL1 carrying pVirG. The different constructs cultures were grown in LB medium supplemented with 75 mg/L choramphenicol and 50 mg/L kanamycin and 4 mg/L tetracycline for *Pi-Avr2, Avr3a and R3a*, with 100 mg/L spectinomycin, 75 mg/L chloramphenicol and 4 mg/L tetracycline for *Avrblb1*and *Avrblb2*. Inoculum of the first experiment was prepared using the growth of bacteria in LB media (15 gr/L Agar, 10 gr/L Bacterial Peptone, 10 gr/L NaCl and 5 gr/L Yeast Extract) until stationary phase and subsequently spinned down at 3600 RPM. Bacteria were resuspended in MMA media (10mM Acetosyringone, 10mM MES and 5 gr/L MS salts) In the second experiment the bacteria were precultured in 3 ml LB, where after they were transferred to YEB medium(5 gr/L Beef Extract, 1 gr/L Yeast Extract, 5 gr/L sucrose, 5 gr/L Bacterial Peptone and 2mM MgSO4) and allowed to grow to OD_{600} 0.4-2.0, afterwards they were spinned down at 3600 RPM for 10 min. and subsequently resuspended in MMA medium. Individual constructs were prepared at an OD_{600} of 0.2 and the combination *Pi-Avr3a /R3a* at 0.3 were used to infiltrate 3 to 5 weeks old wild species plants, reaching their 9th leaf stage (Vleeshouwers and Rietman 2009). Specific recognition of the *Avr*-gene by a putative *R* gene present induces hypersensitive cell death response (HR) from 5 days post-infiltration. Response intensity was scored in percentage of

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infiltrated area showing cell death. Zero to 20% of dark lesion were characterized by a (-), 21 to 35% by (+), 36 to 65% by (++) and 66 to 100% by (+++).

Geographical localization of the accessions

Geographical origin (latitude, longitude) of the accessions was retrieved from the CGN website (www.cgn.wur.nl) and from literature (Rodriguez and Spooner 1997; Hijmans et al. 2007). The desktop application Google Earth (GE) was used to visualize the location of the genotypes. Different tags were associated to genotypes containing *Rpi-blb1*, *Rpi-blb2*, *Rpi-blb3* or their combinations.

We used the internet application SUPRAmap (http://supramap.osu.edu; Janies. 2007) in order to associate the different haplotypes with their geographic distribution. The out-group for the *Rpi-blb1* and *Rpi-blb3* was assigned by performing a phylogenetic analysis in Mega Software (version 4), using the 'minimal evolution' option with standard parameters. The classification described in Table 1a and 1c was used to illustrate the geographical localization of groups B and C (for Table 1c) and the M1 clade from Table 1a is highlighted to illustrate the spread of the M1 allele (see supplementary KMZ file).

ACKNOWLEDGEMENTS

We would like to thank dr. Danial Janies (institute, country) for his kind help and access to the SUPRAmap website. We are grateful, to the Scottish Crop Research Institute (SCRI) and The Sainsbury Laboratory (TSL) for giving us the opportunity to use the effector *Pi-Avr2* and *Avrblb2* respectively; and to Nicolas Champouret (Wageningen UR Plant Breeding) for cloning *Avrblb2* into the expression vector. We acknowledge P.R.J. Birch, G. Kessel and F. Govers for providing *Phytophtora* isolates. Funding for this project was provided by the EU (contract No. FOOD-CT-2005-513959 BIOEXPLOIT) for the Bioexploit project and a grant from the Wageningen University Fund.

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Supplementary Table S2: Characteristics of the set of *P.infestans* isolates used in this study

Isolate	Year	Geographic origin	Mating type	Obtained from	Virulence profile	Reference
PIC 99177	1999	Metepec, Mexico	A2	Kessel, PRI, WUR	1.2.3.4.7.9*.11	Flier et al. 2002
PIC 99189	1999	Metepec, Mexico	A2	Kessel, PRI, WUR	1.2.5.7.10.11	Flier et al. 2002
PIC 99183	1999	Metepec, Mexico	A2	Kessel, PRI, WUR	1.2.3.4.5.7.8.10.11	Flier et al. 2002
IPO-C	1982	Belgium	A2	Kessel, PRI, WUR	1.2.3.4.5.6.7.10.11	
90128	1990	Geldrop, Netherlands	A2	Govers, Phytopathology WUR	1.3.4.7.8.10.11	Vleeshouwers et al. 1999
UK7824	1978	United Kingdom	A1	Govers, Phytopathology, WUR	1.2.3.6.7	

Supplementary Table S3. Overview of primers used for the screening of the wild potatoes species

Marker	PCR primer (5′ to 3′)	Tma	Fragment lenght	Reference
Blb1 F/R	F: AACCTGTATGGCAGTGGCATG	58 °C	820 bp	Wang et al. 2008
	R: GTCAGAAAAGGGCACTCGTG			
1/1'	F: CACGAGTGCCCTTTTCTGAC	50 °C	213 bp	Colton et al. 2006
	R: ACAATTGAATTTTTAGACTT			
1521/518	F: GAAAGTCTAGAGTTACACTGG	58 °C	704 bp	Wang et al. 2008
	R: CAATCACAATGGCAGGAACC			
517/1519	F: CATTCCAACTAGCCATCTTGG	58 °C	750 bp	Wang et al. 2008
	R: TATTCAGATCGAAAGTACAACG			
RGA1F/R	F: CAGTCACTTTCTTGTTTGCCG	55 °C	845 bp	Wang et al. 2008
	R: CAGTAGTGAAGTCACTGTGTG			
Blb2F/R	F: GGACTGGGTAACGACAATCC	58 °C	773 bp	Wang et al. 2008
	R: AGCACGAGTTCCCCTAATGC			
Blb3F/R	F: TGTCGCTGAAAGAGTAGGCC	50 °C	618 bp	
	R: TATGGAGTGGCTTCTTGAAC			
	Ra: TCCTGAGGTTTATATGACTCATC		757 bp	

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Chapter 4

Phytohthora infestans resistance gene *Rpi-mcd1.1* from the Argentinean *Solanum microdontum* is functionally distinct from other *R2* homologues of Mexican origin

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Anoma A. Lokossou, Vivianne G. A. A. Vleeshouwers, Edwin A. G. van der Vossen, Richard G. F. Visser, Evert Jacobsen

ABSTRACT

Wild *Solanum* species are an important reservoir of disease resistance (*R*) genes, which are under constant evolutionary pressure amongst others via recombination events between alleles. The major Late Blight (MLB) resistance locus on linkage group IV harbors *R2* homologues isolated from *Solanum bulbocastanum*, *S. demissum*, *S. edinense*, *S. hjertingii* and *S. schenckii* that all originate from Mexico. Here we report about the cloning of the NBS-LRR gene *Rpi-mcd1.1* which belongs to the same MLB locus in *S. microdontum* originating from Argentina. *R2* allele mining in the resistant parent RH90-038-21 resulted in 27 homologues among which *Rpi-mcd1.1* was identified. *Rpi-mcd1.1* shares 91% nucleotide identity with *R2* and polymorphic nucleotides are mainly located in the LRR region. *Rpi-mcd1.1* is a unique member of the *R2* family since it provides a resistance that is functionally different from the resistance conferred by the Mexican *R2* gene family. The analysis of recombinant genotypes and the amino acid sequence both reveal multiple recombination events which recently occurred inside the *Rpi-mcd1.1* resistance locus. Phenotypically, *Rpi-mcd1.1* was found to confer qualitative resistance to some *Phytophthora infestans* strains, but quantitative resistance to some other, more aggressive strains. Further characterizations of parents and RH94-076 progeny permitted the identification of two additional *R* genes segregating in the population, namely *Rpi-mcd1.2* also originating from *S. microdontum,* and *Rpi-phu2* originating from *S. phureja.*

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INTRODUCTION

Potato is an important staple food originating from South America and is grown in more than 100 countries, under temperate, subtropical and tropical conditions. About 200 wild potato species, partly domesticated by generations of local farmers, have been collected in South America by different expeditions in the last decades (Hawkes and Hjerting 1969). In the rest of the world, all potato cultivars are derived from the wild species *Solanum tuberosum*. Potato monoculture and the lack of genetic diversity helped the emergence and dominance of late blight disease caused by the oomycete *Phytophthora infestans* (*Pi*).

In general the plant defense system is triggered upon pathogen effector recognition by the plant's resistance genes resulting in cell death of the cells attacked (McHale et al. 2006) and consequently decreasing or even blocking the pathogen's growth. So far, the cloned late blight resistance (*R*) genes belong to the NBS-LRR gene family and *P.infestans* avirulence (*PiAVR*) effectors to the RXLR family. Key domains necessary for R/AVR interactions have been identified in the nucleotide binding site (NBS) and the leucine rich repeat (LRR) domains (Catanzariti et al. 2010; Collier and Moffett 2009; Ellis et al. 2007; Jones and Dangl 2006; Rairdan and Moffett 2006).

The attempt to combat the disease by introgression of *Solanum demissum* resistance genes (R-genes) into susceptible cultivars failed because of the rapid emergence of virulent *P. infestans* isolates, thus resulting in the so-called "broken R-genes". To control the disease, the potato polyculture as applied in the Toluca valley of Mexico and in the Andes inspired modern potato breeders to R-gene pyramiding in cultivated potatoes. Pyramiding is pictured in mixing individual *R-gene* containing cultivars or creating a cultivar containing stacked R-genes. In the attempt to assemble this molecular weapon, additionally to the *Solanum demissum* R-genes, many R-genes were identified and isolated from wild *Solanum* species comprising *S. bulbocastanum* hosting *Rpi-blb1*, *Rpi-blb2*, *Rpi-blb3*, *Rpi-bt1* (Lokossou et al. 2009; Oosumi et al. 2009; van der Vossen et al. 2003; van der Vossen et al. 2005), *S. stoloniferum* harboring *Rpi-blb1* allelic versions *Rpi-sto1* and *Rpi-pta1* (Wang et al. 2008) *; S. venturii* containing *Rpi-vnt1.1, Rp-vnti1.2* and *Rpi-vnt1.3* (Foster et al. 2009; Pel et al. 2009).

R2 allele mining using homologous primers successfully permitted the cloning of *Rpi-abpt*, *R2like*(Lokossou et al. 2009; Park et al. 2005a; Park et al. 2005b; Park et al. 2005c) and *Rpi-snk1-1*, *Rpi-snk1-2*, *Rpi-edn1-1*, *Rpi-hjt1-1*, *Rpi-hjt1-2* and *Rpi-hjt1-3* (Champouret 2010; Jacobs et al. 2010), originating from *S. demissum*, *S. schenckii* , *S. edinense* and *S. hjertingii*. A focused allele mining study using *Rpi-blb3* specific primers in *Solanum* germplasm identified *S. pinnatisectum*, *S. bulbocastanum* (including some subspecies), *S. hjertingii, S. nayaritense, S. brachistotrichum, S. cardiophyllum* and *S.*

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stoloniferum as containing putative functional *Rpi-blb3* homologues (Chapter 3). All *Rpi-blb3*-like containing *Solanum* species are geographically located in Mexico and Guatemala. The functional *R* genes that confer resistance to *P. infestans* among them, all interact with the cognate *P. infestans* avirulence gene *PiAvr2* (RXLRfam7) (Champouret 2010; Lokossou et al. 2009). Another strategy to control late blight is to identify and introgress chromosome loci harboring genes responsible for quantitative resistance traits. Quantitative resistance loci (QRL) have been largely studied and are believed to provide a more stable durable resistance (Gebhardt 1994; Leonards-Schippers et al. 1994; Oberhagemann et al. 1999), so called horizontal resistance in contrast to the vertical resistance provided by *R* genes. Late blight QRL have been identified in many wild *Solanum* species including the Argentinean wild diploid species *S. microdontum* (Colon et al. 1995; Micheletto et al. 2000; Sandbrink et al. 2000). Interestingly, several of the identified PiQRL mapped to the short arm of linkage group IV (Bradshaw et al. 2004; Bradshaw et al. 2006; Leonards-Schippers et al. 1994; Sandbrink et al. 2000), on the *R2* locus (Hein et al. 2009; Hein et al. 2007). Recent studies determined the quantitative resistance of *S. microdontum* accession BGRC 18302-34 (CGN 21342) to be conferred by *Rpi-mcd1,* which was mapped to the *Rpi-blb3* locus at linkage group IV. *Rpi-mcd1* was characterized as a weak-effect resistance gene and expected to be a member of the NBS-LRR gene family (Tan et al. 2008). Since *Rpi-mcd1* was mapped with a complex race IPO-C that is virulent on known members of *R2* cluster, we were intrigued by the possibility to identify a functionally distinct R gene at the *R2* locus.

In our study, we investigated the late blight QRL *Rpi-mcd1* identified in *S. microdontum*. We characterized the resistance spectrum of the population that segregates for *Rpi-mcd1* (Tan et al. 2008) with genetically diverse *P. infestans* isolates permitting the identification of at least three segregating genes. Allelic mining strategies resulted in the identification of *Rpi-mcd1.1*which was functional in a transient complementation assay with *Nicotiana benthamiana.*

RESULTS

Characterization of the parental and progeny resistance spectra

To functionally characterize the resistance of *Rpi-mcd1*, detached leaves of the parental clones RH90-038-21 and RH88-025-50 and their RH94-076 progeny were inoculated with *P. infestans* isolates IPO-C, IPO-0 and PIC99177. In addition, Blb99-256-3, 707TG11-1, AM3778-16 and

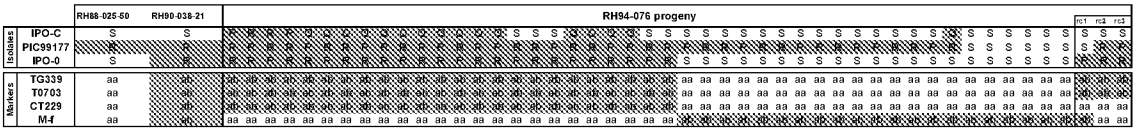
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Cebeco44158-4 from which *Rpi-blb3*, *Rpi-abpt*, *R2-like* or *R2* were isolated, respectively, were included as controls (Table 1 and Fig 1). As expected for typical *R2*or *Rpi-blb3* activity, Blb99-256-3, 707TG11-1, AM3778-16 and Cebeco44158-4 were resistant to IPO-0 and susceptible to IPO-C and PIC99177 (Fig 1). Both clones RH90-038-21 and RH88-025-50 were resistant to PIC99177, with clear cell death lesions at the inoculation spot. However the population segregated into 41 resistant and 7 susceptible genotypes, which suggests that the resistance to PIC99177 is based on 2 (or more) *R* genes originating from both parents. When inoculated with IPO-0, RH90-038-21 was moderately resistant displaying small to large lesions with no or weak sporulation, starting at day 7, whereas RH88-025-50 was susceptible. The resistance segregated in a 1:1 manner in the RH94-76 population, with 27 resistant and 21 susceptible genotypes, suggesting monogenic inheritance of a single dominant *R* gene. In the first experiment, the highly aggressive isolate IPO-C generally caused susceptible phenotypes with large sporulating lesions (Table 1), although previous studies detected *Rpi-mcd1* with this isolate under field conditions. In order to have a better comparison of the resistance phenotypes, we challenged the RH94-076 population plants with the isolates IPO-C and PIC99177, and applied both isolates to the same leaf (Fig.2). In this second experiment, three resistance patterns were observed; tested plants were resistant to PIC99177 and IPO-C, or only to PIC99177, or susceptible to both isolates. None of the tested plants was simultaneously resistant to IPO-C and susceptible to PIC99177. Half of the RH94-76 population displayed clear hypersensitive response (HR)-like cell death lesions at 5 days post inoculation with isolate IPO-C, followed by a low degree of sporulation at 7 dpi, and this was interpreted as quantitative resistance. More than three quarters of the progeny displayed clear hypersensitive response lesions as the parental clones upon PIC99177 inoculation (Table 1). Additionally, there appeared to be a correlation between the resistance to PIC99177 and IPO-C. Champouret et al (2009) described PIC99177 as relatively weak isolate, which can detect a "QRL" or weak R genes in detached leaf assays (DLA). In contrast, for the much more aggressive IPO-C isolate, DLA is considered less appropriate to detect QRL or weak R genes . In line with this, the resistance to IPO-C was mapped under field conditions (Tan et al. 2008). We decided that for our mapping studies using DLA, PIC99177 is an appropriate isolate to use. In conclusion, the genotypes from RH94-76 population which are resistant to the isolates PIC99177, IPO-0 and quantitatively resistant to IPO-C contain the targeted resistance gene *Rpi-mcd1*.

Markers bordering the late blight resistant locus on linkage group IV, TG 339, T0703 and CT 229 (Tan et al. 2008, Park et al. 2005b) fully co-segregate with resistance to IPO-0 PIC99177 and IPO-C with the exception of 1/3 of the PIC99177 resistant offspring in which the markers were not detected (Table 1). This is in accordance with the resistance phenotype of the parental clones RH90-038-21 and RH88-025-50, confirming the presence of two different genes conferring resistance to PIC99177.

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Table 1: Overview of the resistance screening of the parental clones RH88-025-50 and RH90-038-21 and their progeny, the cross population RH94-076. Plants were challenged with three *P. infestans* isolates, IPO-C, IPO-0 and PIC99177. The plants genomic DNA was tested for the presence of the markers TG339, T0703, CT229 and Mf. Abbreviations: S: susceptible; R: resistant.



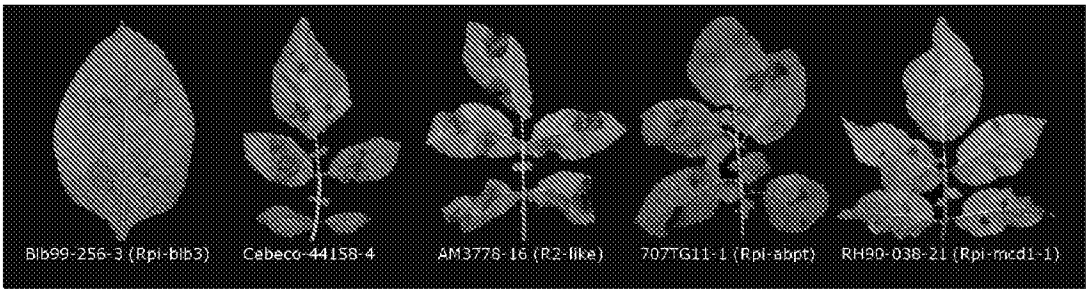


Figure 1: Typical disease phenotypes 8 days after inoculation with a sporangiospore suspension of Phytophtora infestans isolate PIC99177. The genotypes carrying the resistance genes *Rpi-blb3* (Blb99-256-3), *R2* (R2-differential), *R2-like* (AM3778-16), *Rpi-abpt* (707TG11-1) couldn't stop the pathogen proliferation. While the genotype carrying *Rpi-mcd 1-1*(RH90-038-21) showed clear hypersensitive response (HR) thus stopping *Phytophtora* growth.

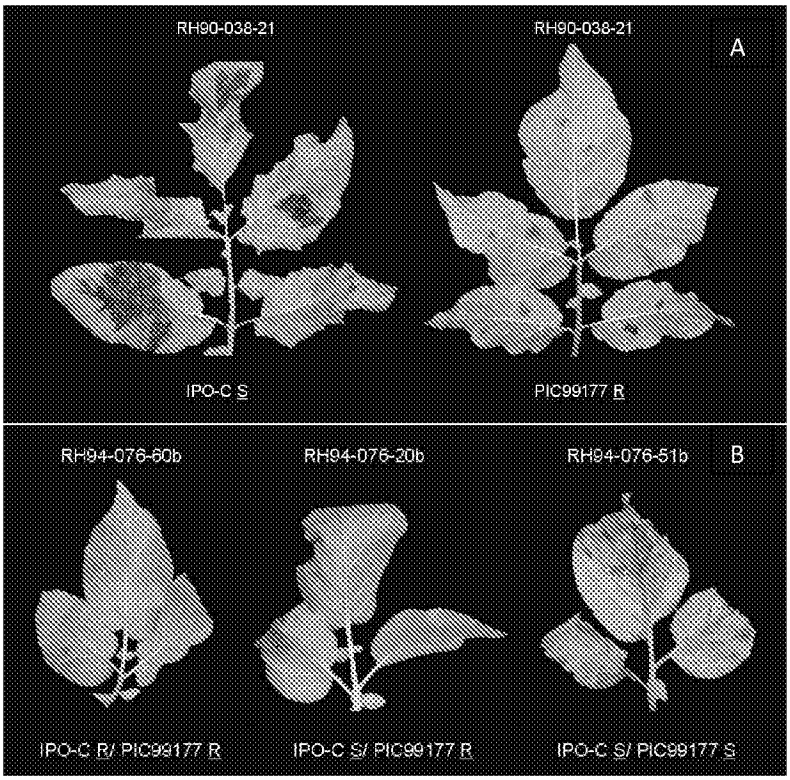


Figure 2: (A) Phenotype of the resistant parent RH90-038-21 with clear susceptibility to IPO-C and resistance to PIC99177 and (B) different possible phenotypes of RH94-076 individuals at seven days post inoculation with *P. infestans* isolates IPO-C and Pi99177. The isolate IPO-C has been inoculated on the left part of individual leaves, and PIC99177 on the right part.

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The parental clones are resistant to the isolate PIC99177 and are derived from *S. microdontum* for RH90-038-21 and from *S. phureja* for RH88-025-50. We deduced that these genotypes carry different independently inheriting genes which confer resistance to PIC99177 that we named *Rpi-mcd1.1* and *Rpi-phu2*, respectively.

In addition, three recombinant genotypes were identified (Table 1). Two recombinant genotypes have resulted from one recombination event which occurred between the marker CT229 and the resistance locus. Remarkably, the existence of the third recombinant genotype can only be explained by the existence of 2 R genes in the locus, *Rpi-mcd1.1* and *Rpi-mcd1.2* conferring resistance to PIC99177 and IPO-0, respectively. The recombination event occurred between the two resistance genes, with *Rpi-mcd1.1* located next to the marker CT229 and *Rpi-mcd1.2* next to the markers TG339 and T0703.

Cloning of Rpi-mcd1.1

Rpi-mcd1.1 belongs to the major late blight resistance locus on LG IV that also harbors *Rpi-blb3* (Tan et al. 2008). In view of the conserved markers CT229 and TG339 we hypothesized that *Rpi-mcd1.1* and *Rpi-blb3* were homologues and thus attempted to clone the gene through an allele mining strategy. The same primers used to amplify the *Rpi-abpt, R2* and *R2-like* (Lokossou et al 2009) candidates were used to amplify full-length *R2GH* from the potato clone RH90-038-21. Amplicons of the expected size were cloned into pDONR221 and fully sequenced. In total, 27 unique sequences were obtained from RH90-038-21 featuring 88 % to 92% nucleotide (nt) identity with *Rpi-blb3* and 89% to 94% nt identity with *R2*.

Phylogenetic analyses using UPGMA algorithm method resulted in the classification of the *R2GH* sequences in three clades (Fig.3). Specific primers differentiating between and within the clades of homologues (Table S2 and Fig 3) were tested on RH94-076 population to search for a correlation with the resistant phenotype observed. The primer pairs M-a and M-d could differentiate the clades I and IV, respectively, but there was no correlation with the resistance phenotype. M-f primer pair is specific to clade II and interestingly, M-f and M-h primer pairs amplified specific fragments which segregate in the progeny of RH94-076 in repulsion phase when comparing to the resistant phenotype (Table 1). This result means that the candidate *R2GH1-18*, *R2GH1-19*, *R2GH1-23*, *pseudoR2GH1-3, pseudoR2GH1-5* and *-pseudoR2GH1-9* originate from the *S. tuberosum* genotype SH 76-128-1857. (Tan et al.2008). Since these candidates are not from *S. microdontum* descent they were discarded from further analyses.

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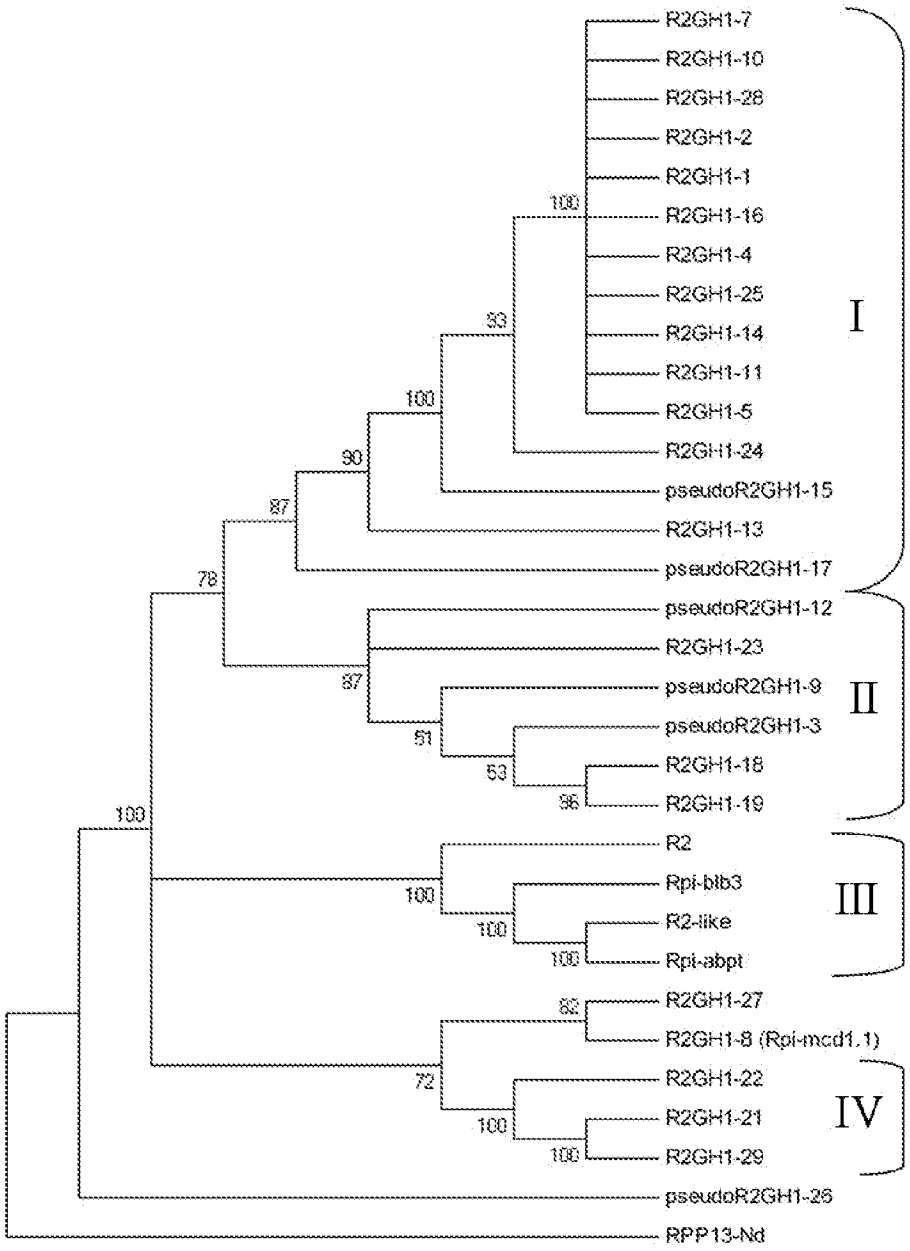


Figure 3: Phylogenetic tree at the nucleotide level of 27 *R2GH* candidates including *Rpi-mcd1-1*and four resistance genes *Rpi-blb3*, *Rpi-abpt*, *R2* and *R2-like* belonging to the same locus on LG IV. RPP13 had been used as an out-group. The clade's numbers are indicated on the right side. UPGMA algorithm with bootstrap analysis of 500 replicates. Numbers above the branches are bootstrap values in percentages.

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Additionally, nucleotide and amino acid sequence analyses identified six pseudo-genes harboring early stop codons in the open reading frame which were also discarded from further studies Finally, the remaining eleven *R2GHs* which are coding for unique amino acid sequences were considered candidates for *Rpi-mcd1.1*.

To study which of the eleven *R2GHs* confers late blight resistance, they were targeted for functional analysis. Candidates were transiently expressed in *N. benthamiana* leaves and further inoculated in DLA with the avirulent *P. infestans* isolates PIC99177 and IPO-C. Phenotypes were observed at seven days post inoculation (Fig 4). No traces of sporulation were observed for both isolates on *N. benthamiana* leaves transformed with *R2GH1-8*, whereas the leaves expressing the other constructs showed large sporulating lesions (Fig S1). *P. infestans* isolate PIC99177 displayed clearly smaller lesion at the inoculation point in comparison to IPO-C, which may be explained by the higher aggression level of the latter strain (Champouret et al. 2009). In conclusion, scoring of the tested leaves indicated that *R2GH1-8* conferred strong resistance to PIC99177 and was therefore designated *Rpi-mcd1.1*.

To confirm complementation by *Rpi-mcd1.1* in potato, stable transformants of potato Desiree were generated. Detached leaves of mature transgenic potato plants were challenged with *P. infestans* isolate PIC99177 and IPO-0. As expected, stable transformed plants containing *Rpi-mcd1.1* were resistant to PIC99177, but susceptible to IPO-0 (data not shown), indirectly confirming the existence of the second R-gene in the locus of the parental plant RH90-038-21, which was named *Rpi-mcd1.2*.

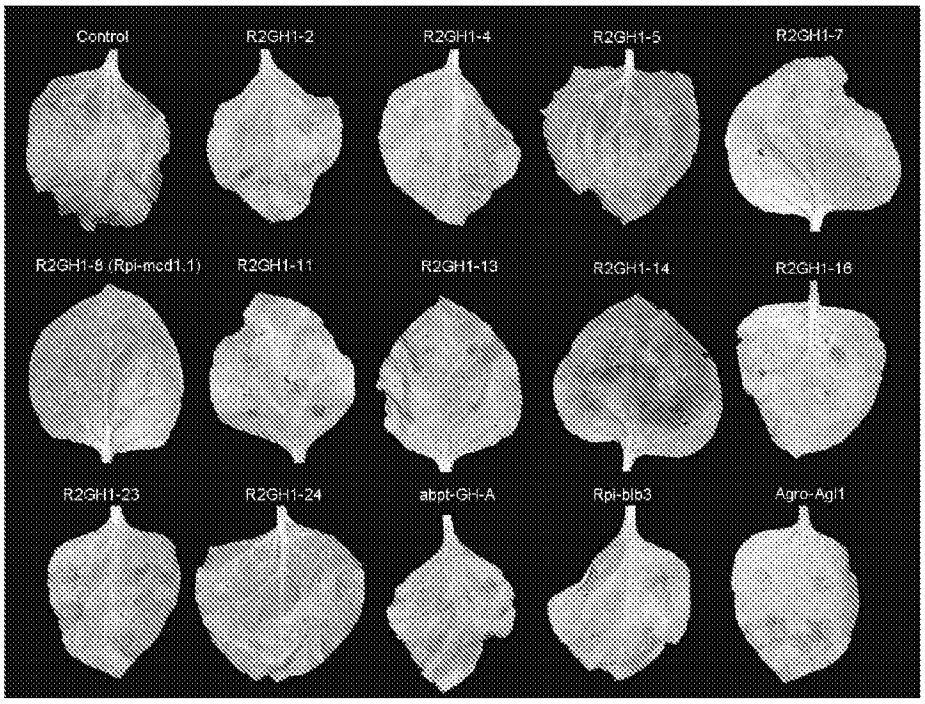


Figure 4: *N. benthamiana* infiltrated leaves with *R2GH*-candidates, the resistance gene *Rpi-blb3* or an empty *Agrobacterium* (Agl1) displaying different resistance phenotypes when inoculated with *P. infestans* isolates IPO-C (left part of the leaf) and PIC99177 (right part of the leaf).

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Molecular structure and recognition specificity

Alignment of the nucleotide sequences coding for complete ORFs, and subsequent analysis of informative polymorphic sites (IPS), i.e. sites where two or more genes carry the same nucleotide (Parniske et al. 1997), reveals a region of 1200 nucleotides residing between kinase 2 and LRR6 (Fig 5) where recombination is increased. Three constant nucleotide structures are present in the *R2GH* pool represented in the clustal W alignment (Fig.5) by *R2GH1-1*, *R2GH1-18* and *R2GH1-21* belonging to clades I, II and IV, respectively (Fig 3). Recombination between these constant structures gave rise to seven different homologues, i.e. *R2GH1-8, R2GH1-22, R2GH1-13, R2GH1-19, R2GH1-23, R2GH1-24* and *R2GH1-27.* Interestingly, *R2GH1-19* and *R2GH1-24* appear to be complementary recombinant genes which arose from a double recombination event in the region of 379 nucleotides between nt positions 1259 and 1638 (Fig 5). *Rpi-mcd1.1* (*R2GH1-8)* appears to be a combination of sequences of the three constant structures plus one unknown gene between the nt positions 1043 and 1259 (Fig 5 and Fig 6). These clear blocks of sequences from different members of *R2GH*s pool indicate that sequence exchange events have been involved in the rise of resistance gene *Rpi-mcd1.1.*

To study the nature of specificity conferred by *Rpi-mcd1.1*, the nucleotide sequences of R2GHs were first aligned with *R2/ Rpiblb3* homologues. The *R2GHs* contain ORFs of 2538-2541 nucleotides that encode proteins of 845-846 amino acids harboring the same signature sequences as R2 homologues. Clustal W amino acid sequence alignment of *Rpi-mcd1.1* together with the three constant structures described above *R2GH1-1, R2GH1-18* and *R2GH1-21*, and with *R2* reveals identical LZ-NBS region with *R2* delimited by the same break point described in the structural analyses of *R2* homologues by Lokossou et al (2009) (Fig.6). *Rpi-mcd1.1* LRR domain differs from *R2* LRR in the polymorphic amino acids which are mainly localized in the xxLxLxxx repeats indicating a putative Pi-Avr differential recognition.

To study *Rpi-mcd1.1* specificity at the functional level, the R-AVR interaction was reconstructed in *N. benthamiana.* The avirulence protein PiAvr2 that is known to evoke cell death responses when co-expressed with R protein of the R2 family was tested for response to Rpi-mcd1.1. *Agrobacterium* strains expressing PiAvr2 were co-infiltrated together with *Agrobacterium* strains expressing Rpi-mcd1.1 in *N. benthamiana* leaves. Co-infiltration of Pivr2 together with R2 or Rpi-blb3 constructs were used as a positive control and single R-gene or PiAvr2 gene infiltration were used as negative controls.

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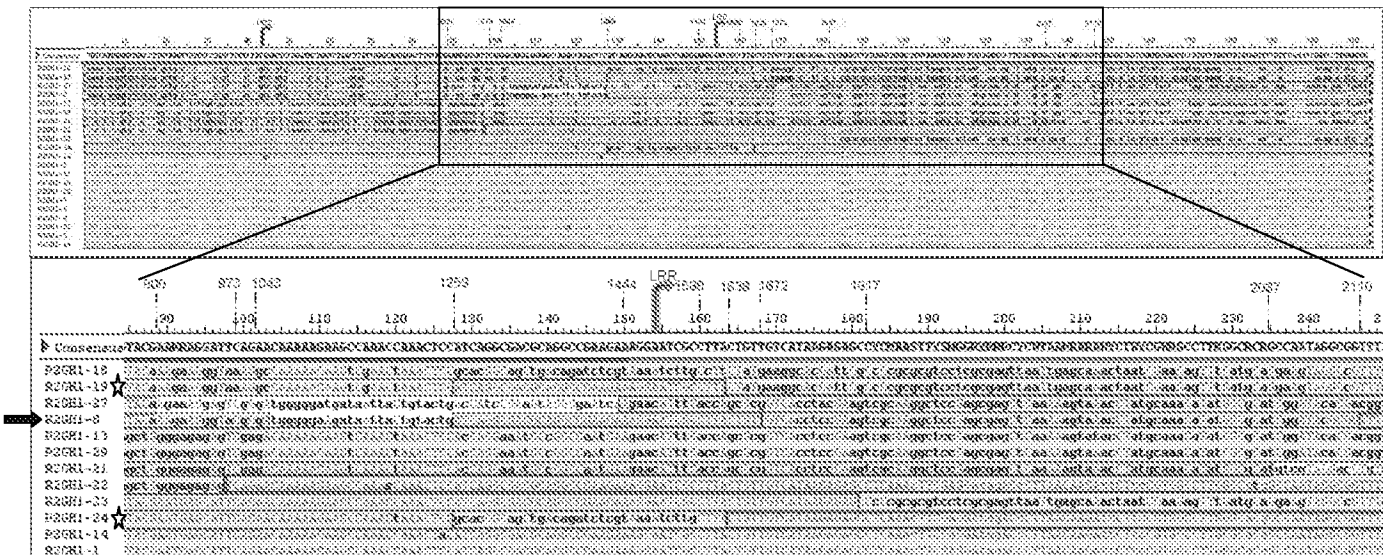
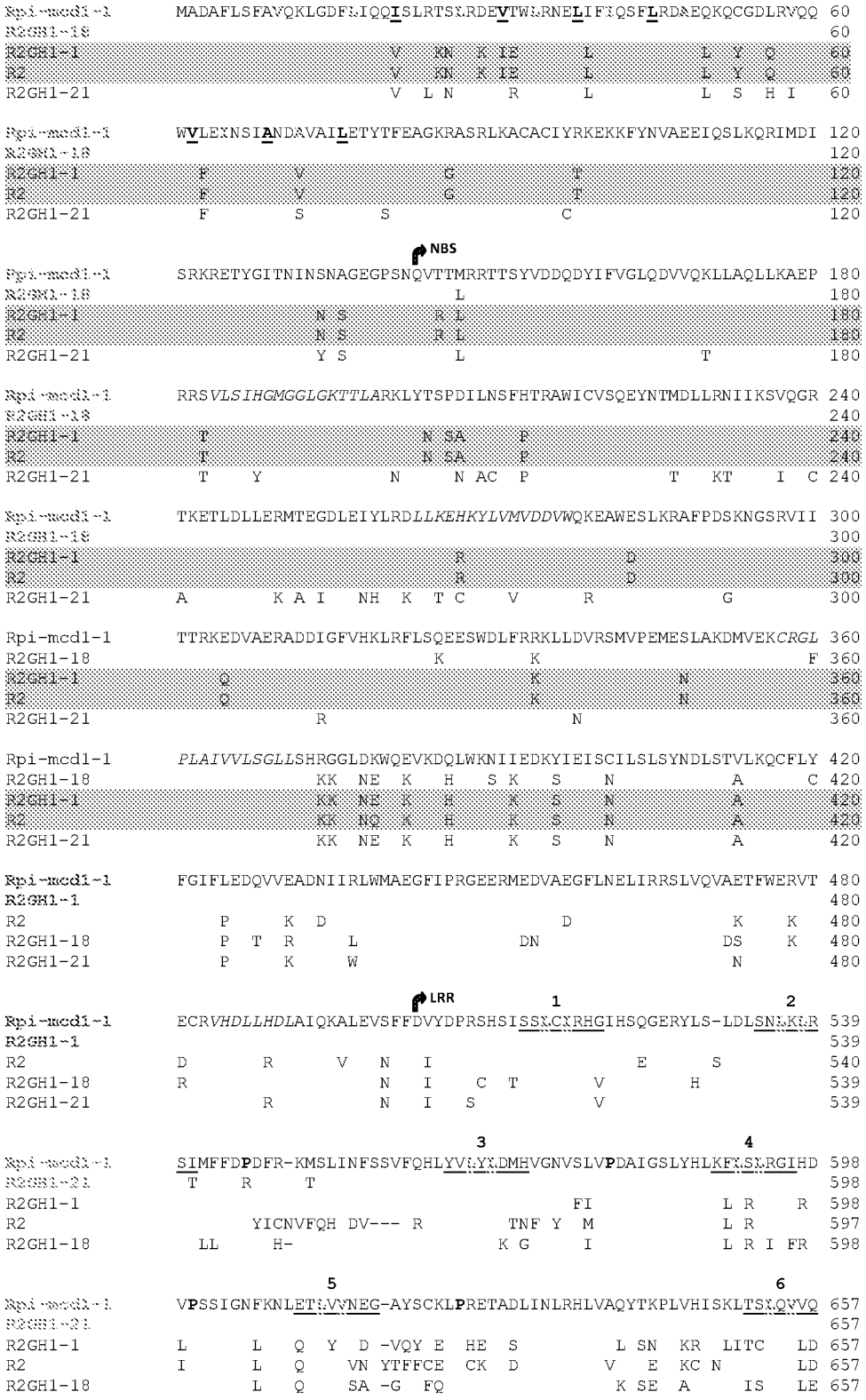


Figure 5: Modular structure analyses of *R2GH* candidates. Sequence of the 314 IPS (Informative Polymorphic Sites) which appear in blue, from the alignment of 21 *R2GH* candidates spanning complete ORFs. The arrow indicates the functional candidate *R2GH1-8* (*Rpi-mcd1-1*). Highlighted in red, green or purple are the sequence blocks referring to R2GH1-18, R2GH1-21 and R2GH1-1, respectively. On the top are indicated the breaking points between the different blocks referring to the original sequence numbering. The figure lights up 3 recombination regions: between [800-1043 bp], [1444-1917 bp], [2037- 2110], and one recombination point at [1259 bp]. *Rpi-mcd-1-1* modular structure is depicted in detail in figure 7. The stars indicate the complementary recombinant *R2GH1-19* and *R2GH1-24*. The beginning of the NBS and LRR regions are indicated by arrows.



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                                        7                                   8
Rpi-mcd1-1      NICCDQWKDVDPVDLVNLRENMDDINKSYSLNNISSLKNLSTNRNFCRGGQSFPDLEFV 717
R2GH1-21                                                          R YVNE   S  717
R2GH1-1         GLG          I         G HN K                              717
R2              GVA                    S  R RS                K I GER   AS  717
R2GH1-18        RV                     R NS K                 T   EDYE   S  717

                      9                        10                        11
Rpi-mcd1-1      NCCEKLQKNWEGRIEKLPHPFPNSITMNVNSLSVLIEDPMPILGILPNLRNNVNFRASY 777
R2GH1-1                                                                     777
R2                     LQ    E   L S     M   F E T        RF      K DG -  776
R2GH1-18               ML  K     DL       M  RK   K              E I  -  776
R2GH1-21               LLL        L       ML EN K T        M       I ED -  776

                             12                        13
Rpi-mcd1-1      EGKEIMCSDNSFSQLEFNINRDLEKLERWHLGTSVMPLIKGNGNNCPNLKEIPERMKDV 837
R2GH1-1                                                                     837
R2                           H    W     D    A         H                  836
R2GH1-18                N    H      V                 D YD                836
R2GH1-21                KN   H          D    A         YE                  836

Rpi-mcd1-1      ERLKRNYML 846
R2GH1-1                 846
R2               L      845
R2GH1-18         Q      845
R2GH1-21         L      845
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Figure 6: Structure and functional analyzes. ClustalW comparing Rpi-mcd1-1 to R2GH1-1, R2GH1-18, R2GH1-21 and R2.The sequence names appear in red, green or purple color when Rpi-mcd1-1 sequence is identical to R2GH1-18, R2GH1-21 or R2GH1-1, respectively. Highlighted in grey are the identical block sequences between R2GH1-1 and R2. The NBS and LRR regions are indicated by an arrow. Conserved domains present in the NBS region are in italic. LRR repeats are underlined and numbered. The amino acid L, I, F, A, M and V from the leucine zipper heptads and the LRR repeats appear in bold red

Specific recognition of *AVR* gene by the *R* gene induces hypersensitive cell death response (HR) from 5 days post-infiltration. In contrast to R2, the Rpi-mcd1.1coinfiltrated with PiAvr2 did not show any cell death (data not shown). This is in line with the disease tests with *P. infestans* isolates that are virulent on plants expressing R2 but avirulent on plants expressing Rpi-mcd1.1, and indicates that *Rpi-mcd1.1* is interacting with other effectors than previously described *R2* homologues from Mexican origin.

DISCUSSION

In this study, we cloned and elucidated the function of one resistance gene, *Rpi-mcd1.1*, originating from *Solanum microdontum* accession BGRC 18302-34 (CGN 21342) collected in northern Argentina. This *Rpi*-gene provides qualitative resistance to some *P. infestans* isolates and a typical hypersensitive reaction was observed after infection with the late blight isolate PIC99177 and IPO-0. To other isolates e.g. IPO-C, a quantitative resistance was observed, in line with previous studies (Sandbrink et al. 2000; Tan et al. 2008). Resistance assays and screening of the closest known markers of linkage group IV in

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the population RH94-076 indicated the presence of at least three *R*-genes, *Rpi-mcd1.1* and *Rpi-mcd1.2* conferring resistance to the *Pi* isolates PIC99177 and IPO-0, respectively, and *Rpi-phu2* conferring resistance to PIC99177. *Rpi-mcd1.1* and *Rpi-mcd1.2* originate from *S. microdontum* and are located on linkage group IV. *Rpi-mcd1.1* was cloned by *R2/ Rpi-blb3* adapted allele mining and conferred resistance to PIC99177 when transiently expressed in *N. benthamiana* leaves and in stable transformants of potato cultivar. Stable transformants confirmed the functionality of *Rpi-mcd1.1* by complementing the susceptibility of the cultivar Desiree when challenged with the isolate PIC99177. No complementation was evaluated against the isolate IPO-0, confirming that the resistance against this isolate comes from a different gene.

Correlation between quantitative and qualitative resistance

Rpi-mcd1.1 is characterized as a "weak" resistance gene by Tan et al (2008) which is in line with the description of PIC99177 by Champouret et al (2009) as a "weaker" isolate than IPO-C or IPO-0. The differences in degree of aggressiveness of these isolates permitted the mapping of *Rpi-mcd1.1* in the field and via detached leaf assay. Additionally, we hypothesize that the quantitative resistance to IPO-C evaluated in the population RH94-076 is also due to different resistance factors including *Rpi-mcd1.1, Rpi-mcd1.2 and Rpi-phu2.* The last one being also characterised as a QRL when the population RH94-076 was challenged to the isolate IPO-C (Dr. C. Celis Gamboa, Wageningen UR Plant Breeding, unpublished results).

Transfer of genes or common ancestor?

The presence of *Rpi-blb3* homologues in *S. microdontum* emphasises the existence of a common ancestor with *S. bulbocastanum* from Mexico. According to Hawkes classification (2004), both belong to the *Petota* section, series *Bulbocastana* for *S. bulbocastanum* and series *Tuberosa* for *S. microdontum*. Using AFLP markers, (Kardolus et al. 1998) unexpectedly found that *S. microdontum* was associated with ser. *Megistacroloba* rather than with ser. *Tuberosa.* Recent phylogenetic studies using AFLP molecular markers classified *S. microdontum* as a distinct genetic unit or species (Jacobs et al. 2008). These results highlight the difficulty to properly classify *S. microdontum* and find phylogenetic links with other *Solanum* species. Our findings shed light on a clear link between the Mexican polyploid *S. demissum* and the Argentinean diploid *S. microdontum*. *S. bulbocastanum* (*Rpi-blb3*), *S. demissum* (*R2*) and *S. microdontum* (*Rpi-mcd1.1*) appear to have a common ancestor but

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different evolutionary forces dependant on their geographical location and the exposure to local *P. infestans* isolates may have shaped functionally distinct R genes for specificity to Avr-genes. *S. bulbocastanum* (*Rpi-blb3*) and *S. demissum* (*R2*) evolved in Mexico and are specifically resistant to P.infestans isolates expressing the effectors *PiAvr2*. In contrast, *S. microdontum* (*Rpi-mcd1.1*) originates from Argentina and probably specifically recognizes different effectors than PiAvr2. Perhaps, an R-AVR pair other than R2-PiAvr2 has driven a co-evolutionary arms race of the Argentinean *Solanum* with local *P. infestans* isolates in the Andes. We hypothesize that the *P. infestans* populations in Mexico and Argentina may have evolved independently, and co-evolutionary forces might have been targeted at different R-AVR pairs. The presence of the effector Pi-Avr2 in isolates might have maintain the LRR structures of *Rpi-blb3* or functional *R2* homologues in Mexico. A survey of the Argentinean late blight population is not yet available in order to relate the presence of Avr-mcd1 with the maintenance of *Rpi-mcd1.1*. in *S. microdontum*.

Intragenic recombination resulted in new recognition specificity of the MLB resistant cluster located on linkage group IV

Rpi-mcd1.1 is so far the only and thus a unique member of the *R2/ Rpi-blb3* cluster providing resistance to the late blight isolate PIC99177. Significant excess of amino acid polymorphism resides in the LRR domain when compared to the one of *Rpi-blb3*, indicating that *Rpi-mcd1.1* probably interacts with another PIC99177-specific effector(s). Similar results were obtained in *RPP13* (*Hyaloperonospora arabidopsidis* resistance gene) allele mining in a diverse set of *Arabidopsis thaliana* accessions (Rose et al. 2004). *RPP13* exhibits the highest reported level of sequence diversity among known R-genes. Hall et al (2009) demonstrated the recognition of the effector ATR13 was mediated by alleles belonging to just a single clade, as it had been reported for *Rpi-blb3*, *R2*, *R2-like* and *Rpi-abpt* (Lokossou et al 2009). Additionally RPP13 alleles in other clades have evolved the ability to detect other *H. arabidopsidis* ATR protein(s) as it might be the case for *Rpi-mcd1.1*. Although certain resistance gene clusters like the one of *Rpi-vnt1* (Foster et al. 2009; Pel 2010; Pel et al. 2009), *Rpi-blb1* (van der Vossen et al. 2003; Vleeshouwers et al. 2008) and *Rpi-blb3* (Champouret 2010; Lokossou et al. 2009), tend to yield only similar genes, it is still worthwhile to further study them in geographically and phylogenetically diverse *Solanum* to find new R genes with distinctive specificity.

Molecular structure studies showed that *Rpi-mcd1.1* has in common five segments with at least three genes present in the gene pool, underlining the role of genetic recombination inside the *R-gene* cluster, resulting in the generation of novel resistance specificities (Hammond-Kosack and Kanyuka 2007;

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Kuang et al. 2004; Meyers et al. 1999; Michelmore and Meyers 1998). Despite the absence of data about the genetic position of each *R2GH* gene, the presence of recombinants in the gene pool implies the close proximity of these genes from each other.*R2/ Rpi-blb3* homologues originating from *S. tuberosum* were identified among the candidate genes isolated from the hybrid *S. microdontum/ S. tuberosum* clone RH90-038-21. Additionally, the presence of a *R2GH* fragment identified as being from the *S. tuberosum* parent, inside *Rpi-mcd1.1* and the identification of complementary recombinant genes demonstrate the occurrence of multiple recent recombination events inside the *Rpi-mcd1.1* resistance locus. The profusion of alleles identified in different studies, 27 alleles in this study, 24 alleles described in Lokossou et al (2009) and 17 alleles isolated by N. Champouret (2010) represent a natural powerful reservoir available for new recombinations and the birth of new resistance alleles in the MLB located on linkage group IV.

MATERIAL AND METHODS

Plant material and late blight isolates

In this study we used the parental clones RH90-038-21, RH88-025-50, and fifty-one new RH94-076 plants from the cross earlier described in Tan et al (2008). The clone RH90-038-21 and the RH94-76 population contain essentially a susceptible *S. tuberosum* genetic background resulting in a germplasm with about 25% *S. microdontum* introgression for the parental clone and less than 12.5% for the progeny (Tan et al. 2008). The clone RH88-025-50 is a hybrid of the *S. phureja* genotype DB-207 and the *S. tuberosum* genotype SH76-128-1865. The characteristics and origin of *P. infestans* isolates IPO-0, IPO-C and PIC99177 are indicated in supplementary Table S1.

Table S1: Race specificity of the different isolates used in this study

Isolate	Year	Geographic origin	Mating type	Obtained from	Virulence profile	Reference
IPO-0	n.d	Unknown	n.d	Kessel, PRI, WUR	0.3b. 4. 7. 10. 11	
IPO-C*	1982	Belgium	A2	Kessel, PRI, WUR	1.2.3.4.5.6.7.10.11	
PIC 99177	1999	Metepec, Mexico	A2	Kessel, PRI, WUR	1.2.3.4.7.9**.11	Flier et al. 2002

* : also known as IPO-82001

** : not conclusive

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Resistance assay

Detached leaf assays were performed as described by (Vleeshouwers et al. 1999). Six days after inoculation, phenotype responses were determined. Leaves showing no symptoms or a localized necrosis at the site of inoculation were scored as resistant and those with clear sporulating lesions (visible by naked eye) as susceptible.

Allele mining

The *Rpi-blb3* allele mining primers Blb3-start and Blb3-end described in Lokossou et al (2009) were used to amplify *Rpi-blb3* homologous ORFs present in the parental resistant genotype RH90-038-21. The *R2GHs* were cloned into the binary vector pKGW-MG between the *Rpi-blb3* promoter and terminator sequences of 2,723 and 883 nucleotides, which were cloned into pDONR P4-P1R and pDONR P2R-P3. The binary clones were subsequently transferred to *Agrobacterium* strain AGL1 and tested in a transient complementation assay in *N. benthamiana,* (Lokossou et al 2009).

Transient complementation in N. benthamiana

Infiltration with *Agrobacterium* strains carrying R2GH were performed in *N. benthamiana.* After the configuration of the constructs were confirmed by PCR, the final expression constructs were then transferred to *A. tumefaciens* strain AGL1 (Lazo et al. 1991) containing the helper plasmid pVirG and pSoup (van der Fits et al. 2000). Recombinant AGL1 *A. tumefaciens* cultures were grown in LB medium (10 g/L bacteriological peptone, 10 g/L NaCl and 5 g/L yeast extract in MQ water) supplemented with 5 mg/L tetracycline , 100 mg/L carbeniciline, 75 mg/L choramphenicol and 100 mg/L spectinomycin. Recombinant *A. tumefaciens* cultures were grown in LB medium supplemented with 100 mg/L spectinomycin. After one or two days a calculated amount of culture (according to OD 0.5 at 600 nm) was transferred to YEB medium (5 g/L beef extract, 5 g/L bacteriological peptone, 5 g/L sucrose, 1 g/L yeast extract, 2 ml/L 1 M $MgSO_4$ in MQ water) supplemented with spectinomycin. After 1 day overnight cells were centrifuged at 3500 rpm and re-suspended in MMA medium (20 g/L sucrose, 5 g/L MS salts and 1.95 g/L MES) supplemented with 1 ml 200 mM acetosyringone to a final OD of 0.2 and infiltrated into leaves of 4 weeks old plants with a 3ml syringe. Three leaves from four plants were infiltrated. After two days, leaves were detached, and spot-inoculated with *P. infestans* strains IPO-C and PIC99177. Seven phenotypes were distinguishable under the binocular and

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were scored from 0 to 6 reflecting the resistance level provided by the tested R2GH to the leaf (Fig S1). Scores of the 24 leaves per construct were computed and the average was calculated.

Agrobacterium-mediated co-expression in *N. benthamiana*

The selected clone *R2GH1-8* was co-expressed with the avirulence effector *Pi-Avr2* cloned in pGRAB vector (kindly supplied by Dr. P. Birch, SCRI. Scotland. UK). Pi-Avr2 clone was transferred to *A. tumefaciens* AGL1 cultures and grown in LB medium supplemented with 30 mg/L choramphenicol and 50 mg/L kanamycin. *Rpi-blb3* was used as a control. The same protocol as the transient complementation was used for the sub-cultures. PiAVR *A. tumefaciens* cultures and R2GH1-8 *A. tumefaciens* cultures were mixed at a final OD600 of 0.1 for each and infiltrated into leaves from 5 to 7 week old plants.

Wild type plant Pi-Avr infiltration was done as described in Vleeshouwers and Rietman (2009). In order to verify that the resistance pathway works, co-infiltration of *Rpi-blb3* and *Pi-Avr2* was used as control.

Transformation of susceptible potato variety

The constructs pKGW-R2GH were transferred in AGL1 (Lazo et al. 1991). After verifying their stability in *Agrobacterium* these clones were transformed to the susceptible potato variety Desiree. Overnight cultures of the transformed *A. tumefaciens* strain were used to transform internodal cuttings from *in vitro* grown plants (Heilersig et al. 2006). A total of 100 to 200 explants were used for each transformation

DNA sequencing, primers design and computer analysis

Sequencing reactions were performed using a dye terminator cycle sequencing reaction kit (Perkin-Elmer, Pt Biosystem, Warrington, UK), M13 universal forward and reverse primers, and an ABI377 automated sequencer (Applied Biosystem, La Jolla, CA, USA). Sequence contigs were assembled using the STADEN sequence and analysis program (Dear and Staden 1991). The binary constructs were sequenced using a primer walking strategy (700 bp by 700 bp). Multiple sequence alignments were conducted using ClustalW2 program accessible from EMBL-EBI web page. Alignments were

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produced using DNASTAR Lasergen Seqman version 8.0.2. The "CLC main workbench 5" program, version 5.0.2 was used to design the specific primers and MEGA4 to produce the phylogenetic trees.

ACKNOWLEDGEMENTS

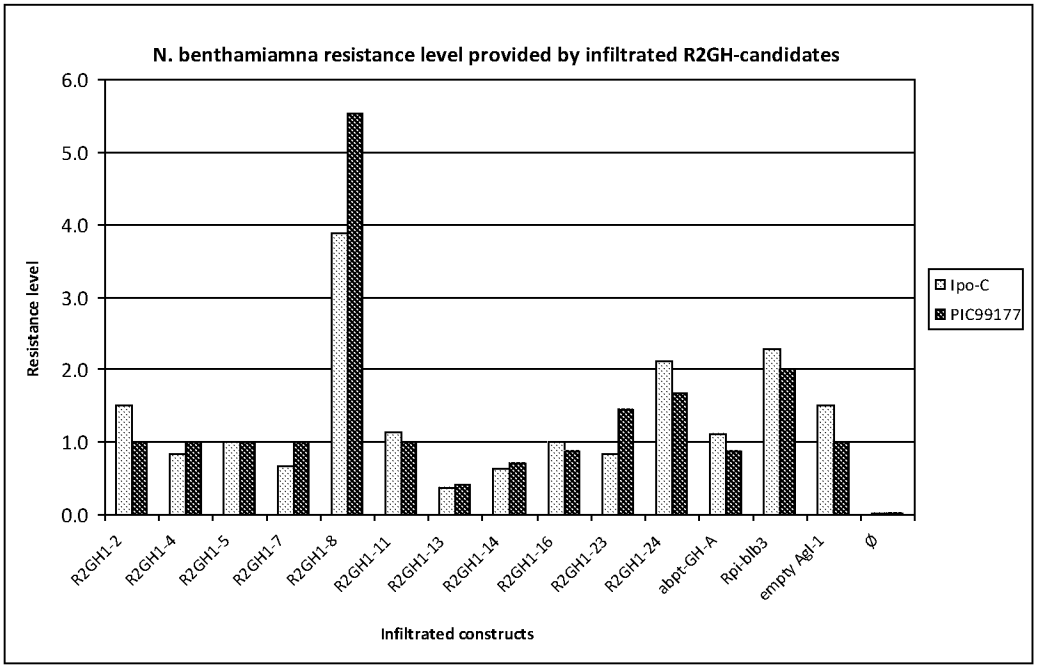
We are grateful, to the Scottish Crop Research Institute (SCRI) and The Sainsbury Laboratory (TSL) for giving us the opportunity to use the effector *Pi-Avr2*. We acknowledge P.R.J. Birch, G. Kessel and F. Govers for providing *Phytophtora* isolates. We would like to thank J. Vossen, M. Arens, H. Rietman and H. Lee for their technical help. Funding for this project was provided by a grant from the Wageningen University Fund.

Table S2: List of primers used in this study

Marker	Primers	sequence (5'----> 3')	Region	Tm C°	Fragment Length (bp)	Specific to
M-a	mcd-Fa	GAGTTCCTTATTCTTSGTGA	2389-2408	61	146	Clade I
	mcd-Ra	TTCAACCGCTCCACGTC	2518-2534			
M-d	mcd-Fd	GAGGATGTCGCTGAAAGAG	922-940	61	1520	Clage III
	mcd-Rd	GTGCCTAAATCCCATCTTTCT	2421-2441			
M-f	mcd-Ff	AAAAACCTTAGCACTCTCAC	2098-2117	61	336	Clade II
	mcd-Rf	ATGCCATCTTTCTACCTTC	2415-2433			
M-h	mcd-Fh	CCAAGAAAGCACTCCAAA	1528-1545	50	469	pseudoR2GH-5b
	mcd-Rh	CACAACCAACCCAATTAAGA	1977-1996			

Primers	sequence (5'----> 3')	Enzyme	Tm C°	Reference
TG339-F	GCTGAACGCTATGAGGAGATG	*Mnl*I	58	Tan et al .2008
TG339-R	TGAGGTTATCACGCAGAAGTTG			
CT229-F	TTGTGAGTGGTGAACTACGGGC	*Hpy*CH4IV	58	Park et al . 2005b
CT229-R	CGGCAATGGTTATGGGAACG			
T0703-F	CCAGTAAGAACAAGCCGATT	*Bme*1390I	54	Tan et al .2008
T0703-R	ATCACCAATTACGCGATCTA			

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0	Heavy sporulation
1	Lesion + heavy sporulation on dead tissue
2	Lesion + moderate sporulation on dead tissue
3	Large lesion and weak sporulation
4	Lesion + weak sporulation on dead tissue
5	Large lesion but no sporulation
6	hypersensitive response

Figure S1: *N. benthamiana* resistance level to Pi isolates IPO-C and PIC99177, provided by infiltrated *R2GH*-candidates and the controls *abpt-GH-A* and *Rpi-blb3* known not to complement susceptibility of *N. benthamiana* to both isolates. Score from 1 to 6 characterize the different phenotypes observed; 1 being susceptible and 6 highly resistant.

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Chapter 5

Exploration of sequence polymorphism in *R2* gene homologues permitted the identification of a single amino acid which triggers the potato/ *P. infestans* interaction R2/PiAVR2

Anoma A. Lokossou, Edwin A. G. van der Vossen, Richard G. F. Visser, Evert Jacobsen

ABSTRACT

The major late blight resistance cluster on linkage group IV has been extensively investigated in various *Solanum* species leading to the identification and/ or isolation of an abundance of late blight (LB) resistance allelic variants. These genes share high percentage identity and define a new *R* gene family characterized by the already known functional *Rpi-blb3*, *Rpi-abpt*, *R2 and R2-like* alleles (Lokossou et al. 2009). Functional LB resistance homologues were identified by susceptible phenotype complementation and/ or effector recognition using the transient co-expression system in *N. benthamiana*. Resistance against a set of *Phytophthora infestans* isolates was coupled with the recognition of the RXLR effector PiAVR2. In our study, we used the sequence information provided by the alleles to identify and functionally characterize the repeats in the LRR domain which are necessary for PiAVR2 recognition. Domain swaps between *Rpi-abpt* or *R2* which interact with PiAVR2 and R2GH-2, which is non functional, permitted the identification of the critical LRR repeats 11, 12 and 13 involved in recognition. Site directed mutagenesis on *Rpi-abpt* pointed to one solvent exposed amino acid in LRR13 crucial for PiAVR2 recognition. These results are an indication of evolutionary differentiation between both partners because of recognition failure by the pathogen.

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INTRODUCTION

The pathosystem formed by the oomycete *Phytophthora infestans* and its hosts among *Solanaceae* is under intensive investigation since the Irish potato famine in 1845. Classical breeding strategies first succeeded in combating Late Blight (LB) by introgression of dominant resistance genes from the wild specie *Solanum demissum* into cultivated potato (Malcolmson 1969; Malcolmson and Black 1966; Müller and Black 1952). The set of eleven *R* genes (*R1* to *R11*) were exploited in monoculture, which besides providing an efficient crop protection, was exerting a strong selection pressure on the pathogen resulting in its rapid adaptation and consequently the defeat of those *R* genes (Fry 2008; McDonald and Linde 2002). A need for new resistance genes appeared and more wild *Solanum* species were investigated for their LB resistance potential. In the last decade, many *R*-genes were identified and isolated from wild *Solanum* species comprising *S. demissum* harboring *R1* (Ballvora et al.), *S. bulbocastanum* hosting *Rpi-blb1*, *Rpi-blb2*, *Rpi-blb3*, *Rpi-bt1* (Lokossou et al. 2009; Oosumi et al. 2009; van der Vossen et al. 2005; van der Vossen et al. 2003), *S.stoloniferum* and *S. papita* harbouring *Rpi-blb1* allelic versions *Rpi-sto1* and *Rpi-pta1* (Wang et al. 2008); *S. venturii* containing *Rpi-vnt1-1* and *Rpi-vnt1-3* (Foster et al. 2009; Pel et al. 2009).

An explanation of the rapid adaptability of *P.infestans* was found in the recent sequencing of its genome which appears to be large and complex with about 74% of repetitive DNA. Annotation of the genome identifies many gene families encoding for effector proteins that are involved in causing pathogenicity. Additionally, comparison with the sequenced *Phytophthora sojae* and *Phytophthora ramorum* showed rapid turnover and extensive expansion of these effector genes (Haas et al. 2009). *P.infestans* effector proteins are classified in two groups, the effectors secreted in the cytoplasm or the apoplasm. The RXLR effectors have been identified among the cytoplasmic secreted proteins and are characterized by an arginine-X-leucine-arginine motif at the N-terminus of the protein that defines a domain required for delivery inside plant cells followed by an acidic region (Jiang et al. 2008; Morgan and Kamoun 2007; Whisson et al. 2007; Win et al. 2007).

Avirulent RXLR effectors (AVR) are detected by the potato plant upon *P.infestans* infection resulting in a hypersensitive reaction stopping the growth of the pathogen (McHale et al. 2006). AVR protein detection is possible in a direct way or indirectly by the resistance genes harboring key domains for pathogen recognition, the nucleotide binding site (NBS) and the leucine rich repeat (LRR) domains (Catanzariti et al. 2010; Collier and Moffett 2009; Ellis et al. 2007; Jones and Dangl 2006; Rairdan and Moffett 2006). Although, the N terminal domain of NBS-LRR protein has been demonstrated to

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play a crucial role in AVR protein recognition (Moffett et al. 2009), multiple studies showed that the LRR domain is the region of the protein that confers recognition specificity (Hulbert et al. 2001; Meyers et al. 1998; Mondragon Palomino and Meyers 2002; Noel et al. 1999). In silico secondary structure of the NBS-LRR protein shows that the LRR repeats with the motif xxLxLxx form a β-strand/β-turn structure in which the solvent x residues are exposed and available for interactions with potential ligands (Kobe and Deisenhofer 1995). These solvent exposed residues are hyper variable and subject to diversifying selection, suggesting that these residues have evolved to detect variation in pathogen-derived ligands (Michelmore and Meyers 1998).

So far six matching pairs of *PiAVR* and potato *R*-gene have been identified, *PiAVR3a/R3a* (Armstrong et al. 2005), *AVR4/ R4* (van Poppel et al. 2008), *AVR-blb1/Rpi-blb1* (Vleeshouwers et al. 2008), *AVR-blb2/ Rpi-blb2* (Oh et al. 2009), *PiAVR2 /R2* (Lokossou et al.) and *AVRvent1 /Rpi-vent1-1* (Pel 2010). Detailed investigation of PiAVR/R interaction was so far orientated to the effector side to identify the crucial domain necessary for the activation of the hypersensitive reaction. The C terminal domains were found indispensable for PiAVR3a and AVR4 recognition (Bos et al. 2006); (van Poppel et al. 2009). Remarkably, PiAVR2 is so far recognized by 10 R genes, *Rpi-blb3*, *Rpi-abpt*, *R2*, *R2-like* (Lokossou et al.) *Rpi-snk1-1*, *Rpi-snk1-2*, *Rpi-edn1-1*, *Rpi-hjt1-1*, *Rpi-hjt1-2* and *Rpi-hjt1-3* (Champouret 2010).

In this study, we explore the information provided by the alleles generated in different *Rpi-blb3* allele mining strategies and use the transient co-expression system in *N. benthamiana* to identify and functionally characterize the repeats in the LRR domain which are necessary for PiAVR2 recognition. Domain swaps between R genes interacting or not with PiAVR2 permitted the identification of a region spanning three LRR repeats of the cloned *Rpi* genes that determine the *R2* resistance specificity. Site directed mutagenesis pinpointed one solvent exposed amino acid to be crucial for PiAVR2 recognition.

RESULTS

Allelic variation

A total of 75 full length amino acid sequences identified on linkage group IV were retrieved from the public nucleotide sequence database and this thesis. These alleles originated from *Rpi-blb3* or *R2* allele mining in *S. bulbocastanum*, *S. microdontum*, *S. demissum*, *S. schenckii*, *S. edinense*, and *S.*

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hjertingii. A blast research on the public nucleotide sequence database resulted in the identification of three BAC clones from the *Solanum tuberosum* diploid genotype RH89-039-16 and two BAC clones from tomato. All BAC clones are mapped on linkage group IV. Annotation of the BAC sequences for *Rpi-blb3* like genes resulted in 5 genes present on the *S. tuberosum* BACs and 4 genes in the tomato BACs, spanning full length ORF. The corresponding protein sequences are classified in a phylogenetic tree into 12 clades (Fig 1). Functional resistance genes interacting with PiAVR2 share 95 to 100% identity with each other, and are bulked in clade X. The functional gene *Rpi-mcd1-1* which doesn't interact with PiAVR2, belongs to clade VI. The genes isolated from wild *Solanum* species which are non functional and do not interact with PiAVR2 share 88 to 99% identity with the functional genes belonging to clade X. The genes identified in *S. tuberosum* and tomato BAC clones share 78 to 89% identity with *Rpi-blb3* and are bulked in clade XII.

Interestingly, three non functional genes, *R2GH 2*, *R2GH-D3* and *R2GH-65*, share 95 to 99% amino acid identity with *Rpi-abpt* or *R2*. In an attempt to identify the causes of non functionality, nucleotide sequences were aligned by clustal W and informative polymorphic sites were identified in comparison to *R2* (Fig 2). R2GH-65 is almost identical to R2 in the exception of a region between the nucleotide positions 693 and 1338, comprising the kinase 2 and glpl domain, which confirm that disruption of the NB-ARC conserved motifs alters the functionality of the gene. The same explanation for non functionality can be given for the polymorphism found in the leucine zipper and NB-ARC domains of the R2GH-6 sequence.

Identification of the LRRs of R2 required for PiAVR2 specific interaction

Remarkably, the R2-GH-2 nucleotide sequence is almost identical to *Rpi-abpt* except in a region comprising LRR11 to LRR13 (Fig 2). Comparison of the amino acid sequences confirms that the majority of the R2GH-2-specific amino acids are concentrated in the putative solvent exposed residues of LRR11-LRR13 (Fig. 3), suggesting that the corresponding LRRs in the functional genes play an important role in determining *R2* specificity.

To analyse the role of LRR11-LRR13 in the interaction with PiAVR2, chimeras were constructed between *R2* and the non-functional *R2GH-2* allele. The unique but conserved restriction sites *Spe*I (located between LRR6 and LRR7), *Bsu*316 (located between LRR11 and LRR12) and *Bsg*I (located between LRR12 and LRR13) were used to swap 5' and 3' ends of the two homologues (Figs. 3 and 4). The constructs were cloned under *Rpi-blb3* regulatory elements and tested by agro-infiltration together with PiAVR2.

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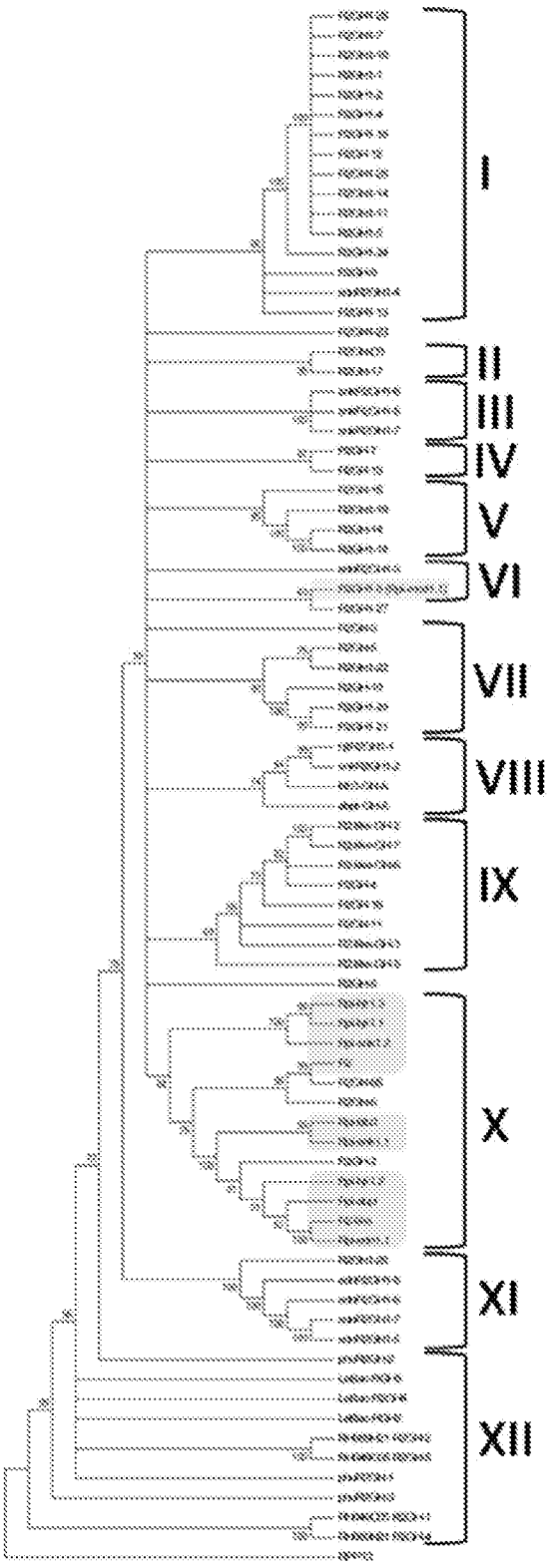


Figure 1: Phylogenetic tree of 75 amino acid sequences of Rpi-blb3 like genes. Functional genes interacting with PiAVR2 are highlighted in blue; the one not interacting with PiAVR2 is highlighted in green. The clade numbers are indicated on the right side. RPP13 had been used as an out-group. UPGMA algorithm with bootstrap analysis of 500 replicates. Numbers above the branches are bootstrap values percentages.

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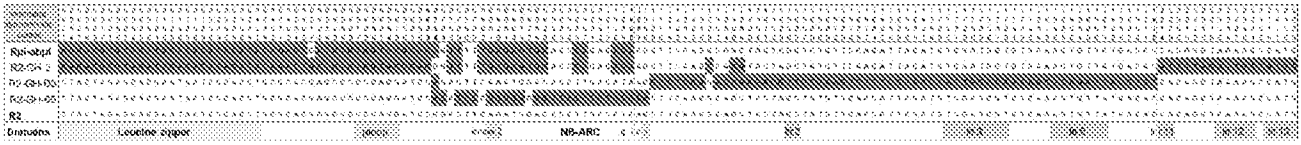


Figure 2: Nucleotide polymorphism analysis of *R2* and 4 homologues sharing informative polymorphic or homologous sequences. In bold are the genes *R2* and *Rpi-abpt* which interact with *PiAVR2*. Only informative polymorphic sites are shown, which reduces the sequence size from 2444 bp to 156 bp. Highlighted in green are the positions polymorphic to *R2* sequence. The vertical number at the top of each row indicates the corresponding nucleotide position in the full consensus sequence, the limits of the recombination regions positioned on the bottom of the figure are indicated in bold. The positions of the different domains characterizing LZ-NBS-LRR genes are indicated down to the sequences.

Table 1: List of primers used for the site-directed mutagenesis

Primer	Sequence (in 5'----> 3' order)	Mismatches	% GC	Tm
lrr11-misLV-F	GATGGTTCTGAGTTT**A**TCAG**T**ACTGACAGAAGATCCGATGC	2	44	78
lrr11-misLV-R	GCATCGGATCTTCTGTCAGTACTGATAAACTCAGAACCATC			
inter-lrr11-12-misIL-F	GAAGATCCGATGCCTATTTTGGGAA**TA**TT**G**CCAAACCTAAGGAATCTC	3	42	78
inter-lrr11-12-missIL-R	GAGATTCCTTAGGTTTGGCAATATTCCCAAAATAGGCATCGGATCTTC			
lrr12-misV-F	GGTTTCCAAACCTAAGGAATCTC**GTT**TTAGATGGAGCTTACGAAGG	3	43	78
lrr12-misV-R	CCTTCGTAAGCTCCATCTAAAACGAGATTCCTTAGGTTTGGAAACC			
lrr12-misK-F	GAAGGTTTCCAAACCTAAGGAATCTC**AAA**TTAGATGGAGCTTACGAAGG	3	41	78
lrr12-misK-R	CCTTCGTAAGCTCCATCTAATTTGAGATTCCTTAGGTTTGGAAACCTTC			
lrr13-misI-F	CTTCAGTCAACTAGAGTTCCTT**AT**TCTTCGTGATCTTTGGAAGC	2	41	78
lrr13-misI-R	GCTTCCAAAGATCACGAAGAATAAGGAACTCTAGTTGACTGAAG			
lrr13-misE-F	GTTCCTTCATCTTCGTGATCTT**GA**GAAGCTAGAAAGATGGG	2	44	78
lrr13-misE-R	CCCATCTTTCTAGCTTCTCAAGATCACGAAGATGAAGGAAC			

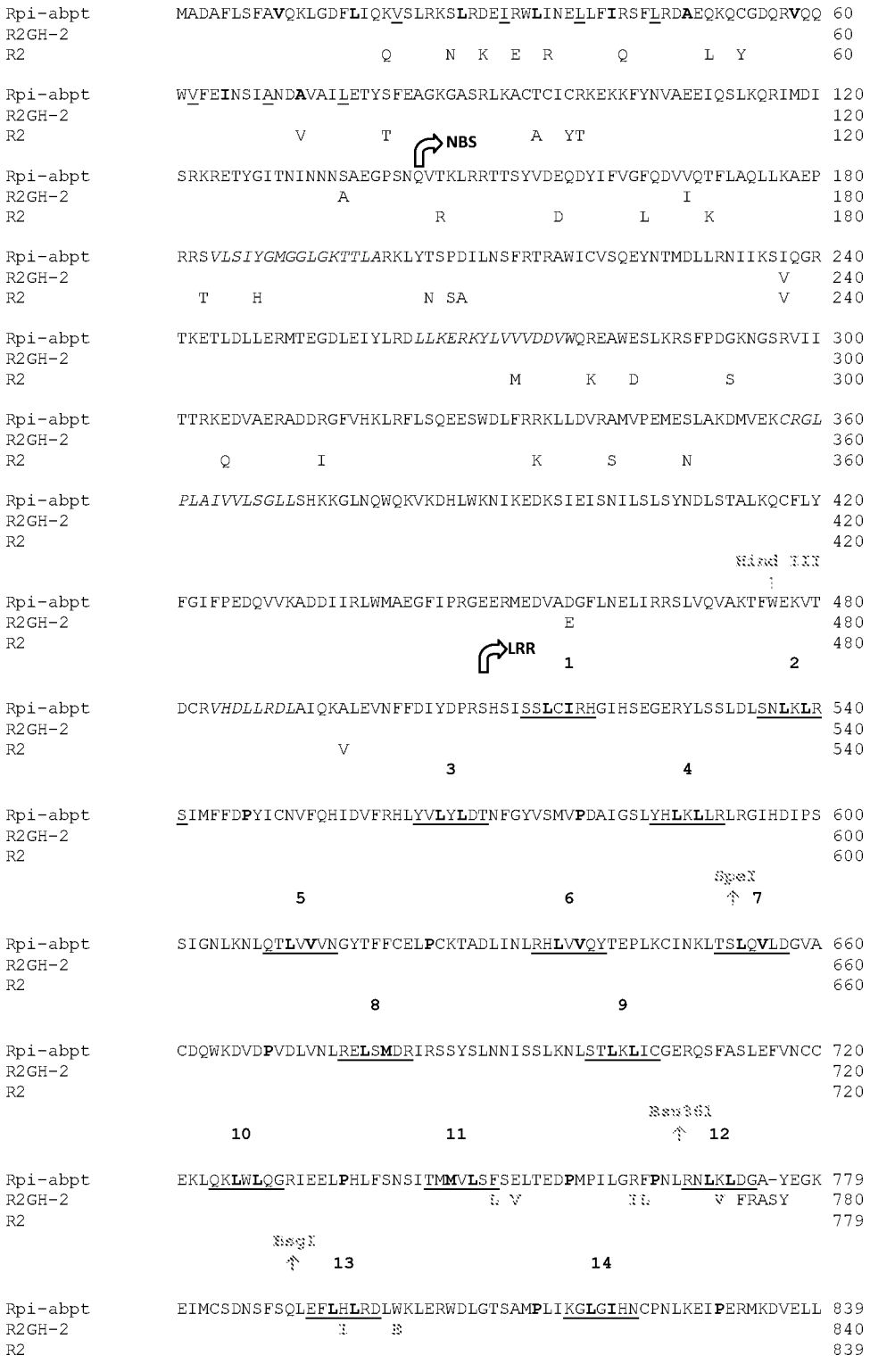
The chimeric constructs R2GH2-SpeI-R2 and R2GH2-Bsu316-R2 were the only constructs able to induce an HR when co-infiltrated with PiAVR2. However, we observed a weaker response with R2GH2-Bsu316-R2 compared to R2GH2-SpeI-R2, suggesting that LRR11, LRR12 and LRR13 are all three involved in the interaction or recognition of PiAVR2. We can also conclude that the two amino acid differences in the NBS domain do not influence the interaction with PiAVR2 (Figs. 3 and 4) while the four amino acids polymorphic between R2 and R2GH-2 in LRR11 influence the recognition but do not suppress the response. However, presence of the R2-specific LRR11, LRR12 and LRR13 alone is not sufficient for R2 specificity, as illustrated by the non-functional homolog R2GH-65, which contains the R2 specific LRR11-LRR13 sequence but differs from the functional genes in the region harbouring NB-ARC conserved motifs. Additionally, R2GH-D3 contains the R2 specific LRR11-LRR13 sequence and differs from R2 in the region harbouring LRR3-LRR10 (Fig 2). The latter observation illustrates the crucial role that putative intra-molecular interactions within the LRR domain and possibly between the LRR domain and the LZ or NBS domain play in determining recognition by the functional Blb3GHs.

R2GH2-Bsu-R2 recognized PiAVR-2 but induced a weaker response than R2GH2-Spe-R2. Our observation indicates that the 4 polymorphic amino-acids in R2GH-2-LRR11 negatively influence but don't completely suppress PiAVR2 recognition. R2GH2-Spe-R2 recognizes PiAVR2 indicating that the 2 SNPs present in the NBS domain of R2GH-2 are not loss of function SNPs. R2-Spe-R2GH2 and Abpt-Spe-R2GH2 are non-functional, indicating that the repeats 11, 12 and 13 are crucial in PiAVR2 recognition. These results are supported by the protein structure prediction of Rpi-abpt and R2GH-2 which show a clear difference of helix, strand and coil structures in the LRR11-LRR13 region (Fig 5).

On the opposite page:

Figure 3: Identification of regions and amino acids crucial for PiAVR2 recognition. Clustal W comparing the amino acid sequences of the functional genes Rpi-abpt and R2 to the non functional gene R2GH-2. The NBS and LRR regions are indicated by an arrow. The unique restriction sites used in domain swapping experiment are indicated in red. Conserved domains present in the NBS region are in italic. LRR repeats are underlined and numbered. The amino acid L, I, F, A, M and V from the leucine zipper heptads and the LRR repeats appear in bold red.

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Rpi-abpt MADAFLSFAVQKLGDFLIQKVSLRKSLRDEIRWLINELLFIRSFLRDAEQKQCGDQRVQQ 60
R2GH-2 60
R2 Q N K E R Q L Y 60
Rpi-abpt WVFEINSIANDAVAILETYSFEAGKGASRLKACTCICRKEKKFYNVAEEIQSLKQRIMDI 120
R2GH-2 120
R2 V T A YT 120
NBS
Rpi-abpt SRKRETYGITNINNNSAEGPSNQVTKLRRTTSYVDEQDYIFVGFQDVVQTFLAQLLKAEP 180
R2GH-2 A I 180
R2 R D L K 180
Rpi-abpt RRSVLSIYGMGGLGKTTLARKLYTSPDILNSFRTRAWICVSQEYNTMDLLRNIIKSIQGR 240
R2GH-2 V 240
R2 T H N SA V 240
Rpi-abpt TKETLDLLERMTEGDLEIYLRDLLKERKYLVVVDDVWQREAWESLKRSFPDGKNGSRVII 300
R2GH-2 300
R2 M K D S 300
Rpi-abpt TTRKEDVAERADDRGFVHKLRFLSQEESWDLFRRKLLDVRAMVPEMESLAKDMVEKCRGL 360
R2GH-2 360
R2 Q I K S N 360
Rpi-abpt PLAIVVLSGLLSHKKGLNQWQKVKDHLWKNIKEDKSIEISNILSLSYNDLSTALKQCFLY 420
R2GH-2 420
R2 420
Kinase IXX
Rpi-abpt FGIFPEDQVVKADDIIRLWMAEGFIPRGEERMEDVADGFLNELIRRSLVQVAKTFWEKVT 480
R2GH-2 E 480
R2 480
LRR
1 2
Rpi-abpt DCRVHDLLRDLAIQKALEVNFFDIYDPRSHSISSLCIRHGIHSEGERYLSSLDLSNLKLR 540
R2GH-2 540
R2 V 540
3 4
Rpi-abpt SIMFFDPYICNVFQHIDVFRHLYVLYLDTNFGYVSMVPDAIGSLYHLKLLRLRGIHDIPS 600
R2GH-2 600
R2 600
SpeI
5 6 7
Rpi-abpt SIGNLKNLQTLVVVNGYTFFCELPCKTADLINLRHLVVQYTEPLKCINKLTSLQVLDGVA 660
R2GH-2 660
R2 660
8 9
Rpi-abpt CDQWKDVDPVDLVNLRELSMDRIRSSYSLNNISSLKNLSTLKLICGERQSFASLEFVNCC 720
R2GH-2 720
R2 720
BamHI
10 11 12
Rpi-abpt EKLQKLWLQGRIEELPHLFSNSITMMVLSFSELTEDPMPILGRFPNLRNLKLDGA-YEGK 779
R2GH-2 V V FRASY 780
R2 779
NcoI
13 14
Rpi-abpt EIMCSDNSFSQLEFLHLRDLWKLERWDLGTSAMPLIKGLGIHNCPNLKEIPERMKDVELL 839
R2GH-2 840
R2 839
Rpi-abpt KRNYML 845
R2GH-2 846
R2 845

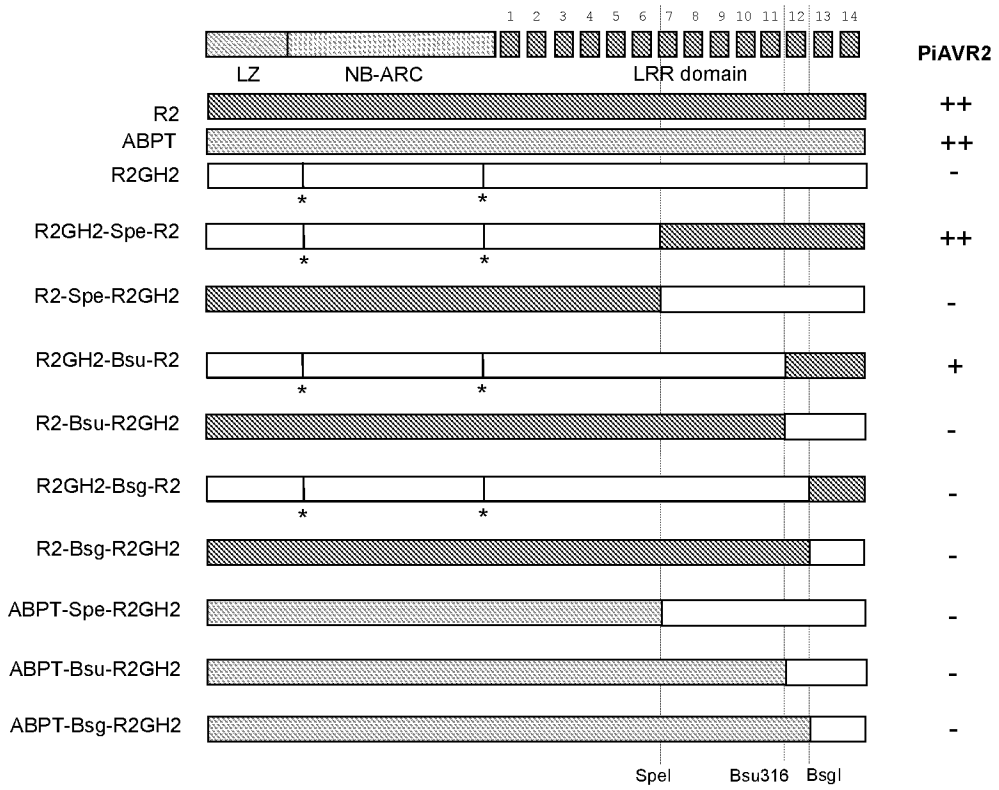


Figure 4: Domain swaps between the functional gene *R2* or *Rpi-abpt* and the non-functional gene R2GH-2. On the top are indicated the different domains (LZ: leucine zipper, NB-ARC and LRR domain with its 14 repeats). Below are indicated the position of the restriction sites used to swap the domains. (*) indicates single nucleotide polymorphism present in R2GH- sequence .in comparison with *Rpi-abpt*. Infiltrated leaves were scored 5 days post infiltration, white or black infiltration spots were scored as (++), yellow pale spots were scored as (+)and no visible cell death region were scored as (-).

Pinpointing of a single amino acid important for PiAvr2 recognition

R2GH-2 is almost identical to Rpi-abpt with the exception of 11 amino acids residing in LRR11, 12 and 13. These amino acids differ from R2GH-2 and Rpi-abpt sequences by their chemical propriety (Fig 3). In an attempt to determine which amino acid(s) in the non functional R2GH2 are critical for the avirulence protein recognition Pi-AVR2, a PCR-based site-directed mutagenesis strategy was used to convert the Rpi-abpt residue individually into the corresponding R2GH-2 residue (Fig 6). Five constructs were made which target seven amino acids individually or in combinations. The construct mut-abpt-1 displays L and V amino acid instead of F and E in the LRR11, mut-abpt-2 has the amino acids I and L instead of R and F also in LRR11, mut-abpt-3 contains the amino acid V instead of K in LRR repeat 12, mut-abpt-4 spans the amino acid I instead of H in LRR13, mut-abpt-5 has the amino acid E instead of W also in LRR13.

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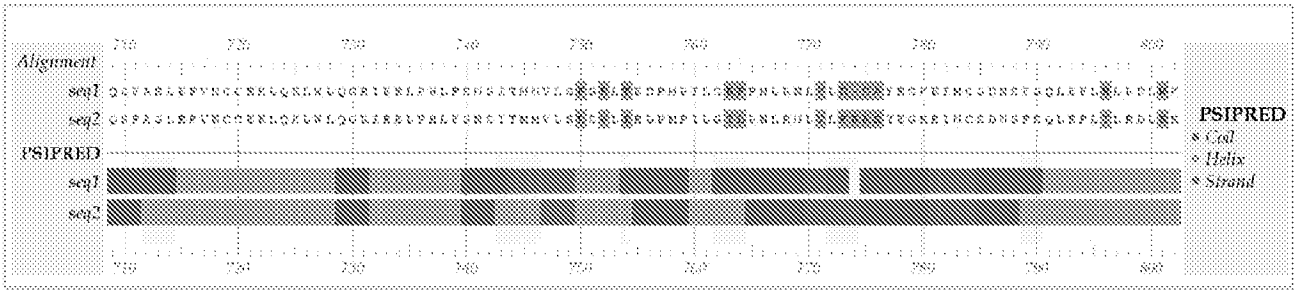


Figure 5: Comparison of the secondary structure prediction of Rpi-abpt (seq1) and R2GH-2 (seq2) in the LRR9 to LRR13 region. Polymorphic amino acids and secondary structure are highlighted in yellow. The figure is a combination of the positional features and differential prediction outputs obtained after analyses of the protein sequences by EPipe 0.922 Server from the center for biological sequences analysis (CBS).

The four constructs mut-abpt- 1, 2, 3 and 5 showed variable intensity of cell death regions. The construct mut-abpt-1 and mut-abpt-2 interact in a less intense reaction with PiAVR2 than the positive control Rpi-abpt. This observation is in correlation with the weak reaction obtained when the chimeric construct R2GH2-Bsu-R2 is co-infiltrated with PiAVR2. Mut-abpt-1 and mut-abpt-2 harbour a double mutation while R2GH2-Bsu-R2 harbours the combination of those double mutations. The constructs mut-abpt-3 and mut-abpt-5 showed a weaker response than mut-abpt-1 and mut-abpt-2. The construct mut-abpt-4 co-infiltrated with PiAVR2 did not induce a hypersensitive response in the *N. benthamiana* leaves. These observations reflect the impact which could have the mutation of strategic amino acids in the structural conformation of Rpi-abpt protein. The mutated amino acids in the constructs mut-abpt-1,2,3 and 5 are identified as residues under strong positive selection pressure (Champouret 2010) with a high posterior probability (p>99%) when the model M8 of the PAML method as applied. The amino acid H in LRR13 displays a posterior probability of 75%. These observations indicate the malleability degree of certain residues like the amino acids K and W belonging to LRR12 and 13, and the crucial importance of others like the amino acid H in LRR13 for *Rpi-abpt* recognition of PiAVR2.

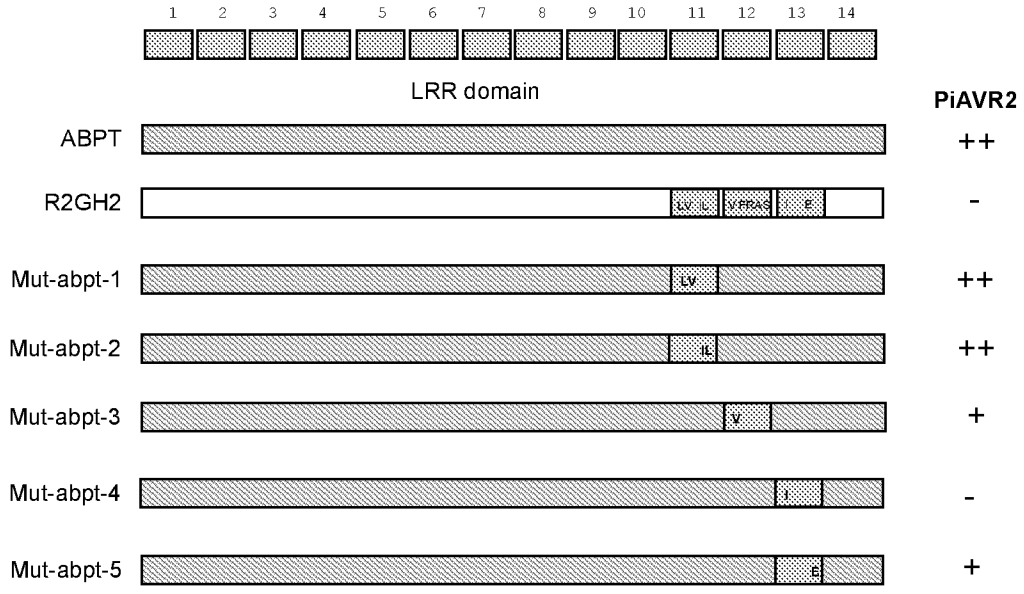


Figure 6: Site-directed mutated constructs tested by co-infiltration with PiAVR2. On the top are indicated the LRR domain with its 14 repeats. Polymorphic amino acids are indicated in comparison with the Rpi-abpt amino acid sequence. Infiltrated leaves were scored 5 days post infiltration, white or black infiltration spots were scored as (++), yellow pale spots were scored as (+) and no visible cell death region were scored as (-).

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DISCUSSION

The major late blight resistance cluster on linkage group IV harbors a profusion of alleles sharing high percentage identity. These alleles define a new R gene family already identified by the cloning of *Rpi-blb3*, *Rpi-abpt*, *and R2-like* (Lokossou et al. 2009). Additionally, N. Champouret (2010) cloned *Rpi-edn*, *Rpi-hjt* and *Rpi-snk* alleles by R2 allele mining. These genes provide resistance against late blight by recognizing the *Phytophthora infestans* RXLR effector PiAVR2. Sequence analyses showed identical C terminal regions starting at the LRR repeat 3 (Lokossou et al. 2009; Champouret 2010). Three non functional R2GHs share high sequence identity with R2 and Rpi-abpt, giving us the opportunity to investigate PiAVR2 recognition specific region(s).

PiAVR2 recognition specificity resides in LRR11, LRR12 and LRR13.

Domain swap experiments have provided direct evidence that LRRs play a major role in determining resistance specificity (Thomas et al. 1997). In depth studies of the *P* and *P2* genes at the *P* locus in flax (Dodds et al. 2001), the *Mi* locus in tomato (Hwang et al. 2000; Hwang and Williamson 2003), and the *Pi2* and *Piz-t* at the *Pi* locus in rice (Zhou et al. 2006) identified essential amino acids in the LRR domain involved in the recognition of the AVR(s) or the functions of the genes. In depth investigation of the tomato Cf-9 disease resistance protein demonstrated that the major specificity-determining amino acids reside at hypervariable solvent exposed positions (Wulff et al. 2009). Here we demonstrate that LRR11, LRR12 and LRR13 are important for the interaction of R2 with PiAVR2. Further more seven amino acids were pinpointed in site directed mutagenesis experiments resulting in the identification of one of the crucial amino acids determining Rpi-abpt and its homologues specificity. The identified Histidine-795 is located in a solvent exposed position of LRR13 (xxLHLxx). Mutation of this amino acid into an Isoleucine as in R2GH-2 resulted in non recognition of PiAVR2. Our results support the positive selection identified in LRR 11, 12 and 13 by N. Champouret (2010) when analyzing 17 *R2* variants using the PAML method. The Histitine-795 had a posterior probability value lower than the other mutated residues reflecting its reduced potential to mutate and maintain the recognition of PiAVR2. This indicates the R protein potential of adaptation to PiAVR conformational change. Modification in the R protein structure might occur in a slow process of searching for the perfect match by trial and error, thus resulting in the profusion of alleles indentified in our allele mining strategy

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Type I and II evolution at the MLB locus on linkage group IV

Sequence analyzes of the 24 *Rpi-blb3/R2* and 27 *Rpi-mcd1-1* homologues (Chapter 2 and 4) reveal a modular structure of the genes which evolution was dominated by sequence exchange, an evolutionary pattern typical for a Type I resistance gene as described for *R*-genes in natural lettuce populations (Kuang et al.). Investigation of the 17 *R2* variants isolated by N. Champouret et al (2010) shows residues in the LRR domain under positive selection thus submitted to single mutation like Type II resistance genes (Kuang et al.), with the aim to counter act to *P. infestans* escape. In our study, we demonstrated that a single mutation on a solvent exposed residue can lead to the lack of recognition of the cognate effector. This could also be interpreted as an adaptation of the gene to recognize an evolved version of the effector, or by the presence in the germplasm of R genes ready to protect the plant against a sudden change in the effector structure. This simultaneous evolution in host and pathogen for survival is also reflected in *Rpi-blb3* allele mining in *S.bulbocastanum* and related species (Chapter 3).

Non functionality or differential P. infestans resistance spectrum or differential pathogen recognition

Fifteen % of the full length homologous proteins reproduce the expected LB resistance spectrum. Thus, the other homologues remain with undetermined functionality. The resistance spectrum covered by *Rpi-mcd1-1* is different from the R2 spectrum and proves the existence of R2 homologues with different recognition specificity (Chapter 4). The demonstrated synteny between potato and tomato (Bonierbale et al. 1988; Gebhardt et al. 1991), and the identification of multiple pathogen resistance genes at one locus open the debate of the existence of homologues genes conferring resistance to other pathogens than *P. infestans* among the *Rpi-blb3/R2* homologue gene collection. Such cluster diversity has been found at the Mi-1/ Rpi-blb2 locus on LG VI which shares 82% protein sequence identity (van der Vossen et al.). *Mi-1* originates from tomato and confers resistance to three species of root knot nematodes (*Meloidogyne* spp.) as well as to the potato aphid *Macrosiphum euphorbiae* (Milligan et al. 1998; Rossi et al. 1998; Vos et al. 1998), and to both B and Q biotypes of whitefly *Bemisia tabaci* (Nombela et al. 2003). *Rpi-blb2* originated from potato and conferred resistance to late blight. *Tm2*, which provides resistance to tomato against the *Tomato mosaic virus,* shares 75% amino acid identity with the late blight resistance gene *Rpi-vent*, both are located on LG IX (Lanfermeijer et al. 2003; Pel et al. 2009). The tomato gene *I2* and potato gene *R3a* which are on LG XI share 88% identity and provide resistance against the fungi *Fusarium oxysporum* and *P. infestans*, respectively (Huang et al. 2005; Ori et al. 1997; Simons et al. 1998). Resistance against the

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potato virus X and the potato cyst nematode *Globodera pallida* are provided by *Rx1* and *Gpa2*, respectively. Both are located on LG V and share an overall homology of over 88% amino-acid identity (Bendahmane et al. 1999; Rouppe van der Voort et al. 1999; van der Vossen et al. 2000). A way to reveal the putative functionality of the *R2/Rpi-blb3* homologues would be to create a library of potato transformants harboring the different alleles and test them with a differential set of *P. infestans* and other pathogens.

MATERIEL ANS METHODS

Domain swaps

pDONR221 constructs harboring *R2*, *Rpi-abpt* and *R2GH-2* were digested according to the manufacturers instructions with the restriction enzymes *Spe*I (Fermentas, Germany), *Bsu*316 (Fermentas, Germany) or *Bsg*I (New England Biolabs, The Netherlands). Digested products were separated by agarose electrophoresis and isolated using the QIAQUICK Gel Extraction Kit from Qiagen (The Netherlands). Different combinations of digested fragments were used in ligation reactions with the T4 DNA Ligase (Fermentas, Germany). Chimeric constructs were verified by comparing restriction digestion patterns obtained after digestion with *Bsu*316 and *Nhe*I (Fermentas, Germany). Additional verification was done through sequencing of the 5'-end, 3'-end and chimeric region of the inserts.

Site direct mutagenesis

Site directed mutations on *Rpi-abpt* in pDONR221 vector were generated by PCR with PfuUltra ® High-Fidelity DNA polymerase (Stratagene) and specific primers containing the desired mutations (Table S1).The PCR product were digested with the enzyme Dpn I according to the manufacturer's instruction. The resulted digestion mix was used to transform XL1-Blue super competent cells (Stratagene QuikChange ® II Site-Directed Mutagenesis kit).

The occurrence of the mutation was verified by sequencing each construct.

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Cloning and testing

Confirmed chimeric and site directed mutated constructs were transferred to the destination vector pKGW-MG (kindly provided by Andreas Untergasser) in between *Rpi-blb3* regulatory elements via a multiple LR reaction with the *Rpi-blb3* promotor in pDONR-P4P1R, the *Rpi-blb3* terminator in pDONR-P2RP3, the ORF of interest in pDONR221, and the binary destination, using the protocol of (Untergasser 2006). The final expression constructs were then transferred to *A. tumefaciens* AGL1 (Lazo et al. 1991) containing the helper plasmid pVirG and pSoup (van der Fits et al. 2000). Each construct was tested by co-infiltration with the *P.infestans* avirulence protein Pi-Avr2 in *N. benthamiana* (as described by Lokossou et al.2009). *pKGW-Rpi-abpt* construct co-infiltrated with Pi-Avr2 was used as positive control. Negative controls were the infiltration with the R-gene or the AVR-gene construct alone. The chimeras R2GH2-Spe-R2, R2-Spe-R2GH2, R2GH2-Bsu-R2, R2-Bsu-R2gh2, R2GH2-Bsg-R2, R2-Bsg-R2gh2 and Abpt-Spe-R2GH2, and the site directed mutants mut-abpt-1 to 5 were functionally tested by co-infiltration with PiAVR2. Infiltrated leaves were scored 5 days post infiltration, white or black infiltration spots were scored as (++), yellow pale spots were scored as (+) and no visible cell death region were scored as (-).

DNA sequencing and computer analyses

Sequencing reactions were performed using a dye terminator cycle sequencing reaction kit (Perkin-Elmer, Pt Biosystem, Warrington, UK). Sequence contigs were assembled using the STADEN sequence and analysis program (Dear and Staden 1991). Alignment and phylogenetic trees were produced using "CLC main workbench 5" program, version 5.0.2. Gene structures were predicted using FGENESH++ (Softberry). Multiple sequence alignments were conducted using CLUSTALX 1.81 (Thompson et al. 1997). The search for genes homologous to *Rpi-blb3* was carried out using the Basic Local Alignment Search Tool (BLAST). Secondary structure domains were identified using the beta version of EPipe 0.922 Server from the center for biological sequences analysis (CBS) (BioSapiens Network of Excellence et al. 2008; (Jones 1999); (Tress et al. 2007).

ACKNOWLEDGEMENTS

We are grateful, to the Scottish Crop Research Institute (SCRI) and The Sainsbury Laboratory (TSL) for giving us the opportunity to use the effector *Pi-Avr2*. Funding for this project was provided by a grant from the Wageningen University Fund

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Chapter 6

General discussion

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The research described in this thesis comprises the isolation of five late blight resistance genes clustering in the major late blight (MLB) locus positioned on potato linkage group (LG)IV. A combination of plant and pathogen investigation techniques permitted the dissection of the MLB locus in terms of functionality, molecular interaction, allelic variation, diversification and evolution. This study could serve as an example for future investigation of resistance gene clusters.

A model for resistance gene cluster studies

We used different techniques in our research, starting with classical map based cloning to isolate the genomic sequence of *Rpi-blb3*, including its regulatory elements. Allelic diversity investigation was done by employing an allele mining strategy permitting the isolation of *Rpi-abpt*, *R2* and *R2-like* (**Chapter 2)**, and leading to the additional identification of 22 *Rpi-blb3* haplotypes among *S. bulbocastanum* and related species (**Chapter 3)**. Functionality of new R genes was best demonstrated in stable transformation experiments with suitable (susceptible) potato cultivars. Allelic versions of a specific R gene could better be tested in an alternative functional test, like described in **Chapter 2,** which is a transient expression of R genes in *N benthamiana* leaves followed by inoculation with *P. infestans* isolates. This method was successfully deployed in **Chapter 4** for the identification of *Rpi-mcd1.1*. Detection of known R genes in *Solanum* species under study in **Chapter 3** was possible using a socalled effectoromics approach, where a hypersensitive response, obtained upon agro infiltration of the avirulence gene in the leaves, gave an accurate diagnostic reaction in the presence of a cognate functional R gene. Finally, co-infiltration of R and AVR gene pairs in *N. benthamiana* leaves allowed the accurate identification of critical amino acid(s) for the determination of a particular R2/PiAVR2 molecular interaction **(Chapter 5)**.

Tracing back the origin of the R2GHs

The major late blight resistance cluster on LG IV harbors a profusion of alleles sharing high percentages of identity. These alleles define a new R gene family, first identified by cloning of *Rpi-blb3*, *Rpi-abpt*, *R2* and *R2-like* (**Chapter 2**). *Rpi-blb3* and *R2* originate from *S. bulbocastanum* and *S. demissum*, respectively. Other allele mining studies revealed an identical sequence of *R2-like* in *S. edinense* (Champouret 2010), thus shedding light on its origin. However, the indistinguishable

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sequence of *Rpi-abpt* was isolated from a *S. tuberosum*/ *S. demissum* clone (Mahdi Mortazavian Wageningen UR Plant Breeding personal communication) adding to the complexity of the origin of this gene that we previously had suspected to be derived from one of the crossing parents used for creation of the quadruple species hybrid ABPT, *S. acaule*, *S. bulbocastanum, S. phureja* or *S. tuberosum*.

In Chapter 5 we report about all the *R2GHs* coding for full length protein sequences. The different alleles isolated in this research and in N. Champouret's (2010) study have been identified in *S. bulbocastanum, S. demissum, S. microdontum*, *S. schenckii*, *S. edinense* and *S. hjertingii*. Interestingly, the *R2GHs* recovered from the clone *S. tuberosum*/ *S. demissum* (Cebeco44158-4) described in **Chapter 2** and the *R2GHs* retrieved from the clone *S. tuberosum*/ *S. microdontum* (RH90-038-21) described in **Chapter 4** ended up in the same phylogenetic clades (**Chapter 5;** Fig.1) and shared a high percentage of amino acid identity. The alleles that the two clones possess in common are seemingly originating from *S. tuberosum.* In addition, the R2GHs identified in the *S. tuberosum* clone RH89-039-16 shared the same phylogenetic clade with R2GHs discovered in tomato. Also, a focused allele mining study using *Rpi-blb3* specific primers in *Solanum* germplasm identified *Rpi-blb3* haplotypes in *S. pinnatisectum*, *S. bulbocastanum* (including some subspecies), *S. hjertingii, S. nayaritense, S. brachistotrichum, S. cardiophyllum* and *S. stoloniferum* **(Chapter 3).**

Using diverse *Solanum* classification systems (Hawkes 1994; Jacobs et al. 2008; Spooner et al. 1991), we failed in our attempt to find phylogenic relationship between the various *Solanum* species harboring *R2GHs* and to deduce the ancestral origin of the MLB residing on linkage group IV.

Intergenic recombination events and mutations, directed by positive selection, shaped the resistance locus

Sequence analyses of the genes isolated by *R2* or *Rpi-blb3* adapted allele mining showed the modular structure of this LZ-NBS LRR R gene family. We identified clear sequence exchanges between the different members of the cluster, nicely exemplified by the structure of *R2* which displayed a different N-terminal sequence when compared to other members of the MLB also interacting with the same avirulence effector PiAVR2. In addition, *R2GHs* identified by N.

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Champouret (2010) showed residues in the LRR domain under positive selection. Sequence exchanges and point mutations directed by positive selection appeared to have contributed to the birth of different functional resistance genes identified in this investigation. The same phenomenon is observed in linkage group V, where multiple intergenic recombinations resulted in the unique *RB/Rpiblb1* (Song et al. 2003; van der Vossen et al. 2003). Following the same evolution pattern as *R2GHs*, only point mutations were detected in the *Rpi-blb1* haplotypes identified in **Chapter 3**.

A previous study demonstrates the rare occurrence of meiotic recombination per chromosome arm in potato (Park et al. 2007). In addition, the presence of identical *R2GH* sequences in different species reflects the conservation of the functional gene during the evolution of *Solanum*. We presume from this observation that the evolution process which led to the actual functional resistance genes studied here, preceded the divergence of the *Solanum* species from an apparent common ancestor.

Co-evolution between Phytophthora infestans and Solanum is permanent

P. infestans originated from the Toluca valley, in the central highlands of Mexico where it evolved together with wild *Solanum* species. Mating type A1 and A2 were first localized in Mexico, while only mating type A1 was present in Europe . In the 1980s the A2 mating type appeared, followed by a dramatic increase of genetic variation in *P. infestans* suggested to be caused by sexual reproduction (Drenth et al. 1994; Fry et al. 1992; Goodwin and Drenth 1997). This led to the rapid defeat of *Solanum demissum* R genes introgressed into breeding material. *Rpi-blb3*, *Rpi-abpt*, *R2* and *R2 like* which have been identified in **Chapter 2**, together with *Rpi-snk1-1*, *Rpi-snk1-2*, *Rpi-edn1-1*, *Rpi-hjt1-1*, *Rpi-hjt1-2* and *Rpi-hjt1-3* (Champouret 2010), which originated from Mexican species and all interact with the avirulence effector PiAVR2. In **Chapter 3** we discovered a new *Rpi-blb3* haplotype which did not interact with PiAVR2 and geographically is located in Guatemala. In addition in **Chapter 4**, *Rpi-mcd1-1* which originated from Argentina also did not interact with PiAVR2. These examples demonstrate the adaptation of the *R2/Rpi-blb3* cluster to the local population of the pathogen and reflect the co-evolution occurring between *Solanum* and *P.infestans*. Since a rapid evolution of *P. infestans* is witnessed under the presence of the two mating types, it is expected to observe a similar fast evolution of R genes present in wild *Solanum*.

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Molecular mechanism underlying quantitative resistance

Among the *Solanum* species known to exhibit quantitative resistance, *Solanum microdontum* is well described for its quantitative resistance gene locus (PiQRL), localized on linkage group IV (Sandbrink et al. 2000; Tan et al. 2008). Cytological characterisation of partial resistance is explained by Vleeshouwers et al. (2000) as a less effective hypersensitive response which is possibly caused by an inadequate or delayed recognition of effectors by weak R genes. Additionally, Champouret et al. (2009) categorized *P.infestans* isolates in use for R-gene characterisation, according to their aggressiveness level. **Chapter 4** depicts the dissection of the quantitative resistance map in a population derived from *S. microdontum.* Leaves of susceptible cultivars transformed with *Rpi-mcd1-1* react with a clear hypersensitive response when inoculated with PIC99177 which displays a very low level of aggression, presuming an underlying "weak" R/ Avr gene pair interaction. Taken together, this information contributes to a better understanding of some, previously called, partial resistance factors and gives an example of a suitable way to characterise these partial resistance factors which now appear to be weak R genes.

Future research and breeding advises

Exploring the synteny existing among Solanaceae

Comparison of genomic positions of phenotypically defined disease resistance genes and R gene homologues in two solanaceous crop genera, *Solanum* (potato and tomato) and *Capsicum* (pepper) highlight syntenous resistance loci. These positions harbor genes or QTL providing resistance to various pathogens. For example, the potato linkage group IV which anchors the major MLB studied in this research is syntenous to the tomato chromosome IV loci hosting the *Hero* gene conferring resistance to *G. rostochiensis*. In pepper, the genomic equivalent position is on chromosome V with *phyt1* QRL conferring resistance to *P. capsici* (Grube et al. 2000a). Only 15% of the R2GHs described in **Chapter 5** reproduce the expected late blight resistance spectrum. The remaining homologues stay with undetermined functionality but the demonstrated synteny between the well studied crops tomato, potato, pepper (Grube et al. 2000b) and the less well studied eggplant (Doganlar et al. 2002) opens speculation to the putative resistance provided by some alleles against more different pathogens than *P. infestans*.

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Broad spectrum and durable resistance

The preference of breeders in introgression of resistance loci providing field resistance, and their believe in a different mechanism underlying quantitative resistance is not strongly supported by the findings in this thesis. In **Chapter 3**, we showed that most of the *Solanum* species displaying large resistance spectrums, often host multiple resistance genes. **Chapter 4** revealed that a classical R gene is in fact encoding the previously identified PiQRL on linkage group IV. This new knowledge about the nature and the distribution of R genes in wild *Solanum* species suggests that achievement of durable resistance can be possible by stacking resistance genes with different resistance spectra and which target functionally different Pi avirulence genes.

P. infestans effector forecast

Taking actual efficient decision support systems (Skelsey et al. 2009) as an example and making use of the current knowledge gathered about *P. infestans*, a complementary forecast approach is proposed. Harvest of *P. infestans* spores present in the target potato cultivation region, followed by the characterization of the avirulence effector content would identify the weakness of the pathogen (Zhu et al.2010). Cultivars containing cognate R genes would be recommended for next season planting. Ongoing studies in different laboratories are conducted to understand the natural evolution of the R genes and the Avr genes, in their natural environment. Using this knowledge, better predictions about the level and the duration of resistance will be possible in the future.

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SUMMARY

Potato is consumed worldwide and represents the fourth most important staple food crop after rice and wheat. Potato cultivars display a large variety of color, shape, taste, cooking properties and starch content but are all derived from the same species; *Solanum tuberosum*. Potato breeding is an economic important activity for international breeding companies, but also plays an important role in breaking the circle of poverty for small farmers. In the Andean region, most farmers use many different potato genotypes combined with farming practices transmitted orally over thousands of years.

The most prominent menace to potato production is Late Blight caused by the oomycete *Phytophthora infestans* which destroys leaves, stems and tubers. Differences of breeding methods between the potato grown in South America and in the rest of the world are related to differences in the consequences of Late Blight infection. In the 19^{th,} century, entire potato fields in Ireland were devastated while in South America *P. infestans* proliferation was readily inhibited. This difference is found in the biodiversity reserve such as that of the Chiloé archipelago in Chile where local small farmers cultivate about 200 varieties of native potato. Obviously, the genetic diversity of cultivated native potato acts as a shield against this versatile pathogen. Inspired by this model to solve the problems raised by the extensive use of potato monoculture, large scale growers and breeders need to maintain genetic diversity in the European staple food crops.

In exploring the South American native potato collection, *Solanum demissum* and later on *Solanum bulbocastanum* appeared to be a source of resistance genes (*Rpi*) to *P. infestans*. The *S. demissum Rpi* genes were transmitted to potato breeding clones by traditional introgression breeding. However the fading of their ability in providing effective resistance against Late Blight infection was witnessed within a decade. In the pursuit to provide a hopefully more durable protection in existing potato cultivars, breeding scientists proposed to directly introduce South American native potato *Rpi* genes in modern potato varieties by using a so-called cisgenic approach. This is in contrast to transgenic plants which can contain genes which have originated from non related genera or even different kingdoms. Breeding of cisgenic plants is on its way to public acceptance because of its inherent resemblance to natural crossing and because efforts are made by the scientific community to explain the principles of cisgenesis.

Lessons were learned from the flexibility of *P. infestans* to overcome the effect of newly introduced *Rpi* genes and, therefore, efforts are still ongoing to discover and clone new *Rpi* genes from native

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potatoes. With this in mind, a new family of *Rpi* genes represented by *Rpi-blb3*, *Rpi-abpt*, *R2, R2-like* and *Rpi-mcd1.1* were characterized in clones derived from *S. bulbocastanum*, *S. demissum, S. edinense* and *S. microdontum.* We accomplished in this research the physical isolation of these genes, the molecular characterization of their functionality and the allelic distribution in the Petota collection.

Rpi-blb3, Rpi-abpt, *R2, R2-like* and *Rpi-mcd1.1* belong to the potato linkage group IV and all contain signature sequences characteristic of LZ-NBS-LRR proteins. The closest known *R* gene so far is RPP13 from *Arabidopsis thaliana w*hich shares an amino-acid sequence similarity of 35%. The LRR domains of Rpi-blb3, Rpi-abpt, R2 and R2-like proteins are highly homologous, whilst LZ and NBS domains are more polymorphic with those of R2 being the most divergent. All four Rpi genes recognize the recently identified RXLR effector protein PiAVR2 which is secreted by *P.infestans* in the cytoplasm of plant cells during the infection process. Unlike *Rpi-blb3, Rpi-abpt, R2* and *R2-like*, the *S. microdontum* resistance gene *Rpi-mcd1.1* does not interact with PiAVR2 and provides a different resistance spectrum. *Rpi-mcd1.1* shares 90% nucleotide identity with *Rpi-blb3* and polymorphic nucleotides are mainly located in the LRR region.

The *S. bulbocastanum* haplotypes of *Rpi-blb1, Rpi-blb2* and *Rpi-blb3* were discovered in several Mexican diploid as well as polyploid species closely related to *S. bulbocastanum*. These three resistance genes occurred in different combinations and frequencies in *S. bulbocastanum* accessions and their distribution is confined to Central America. A selected set of genotypes was tested for their response to the avirulence effectors IPIO-2, Avr-blb2 and Pi-Avr2, which interact with *Rpi-blb1*, *Rpi-blb2* and *Rpi-blb3,* respectively, as well as by disease assays with a diverse set of isolates. Using this approach some accessions could be identified that contain novel, yet unknown, Late Blight resistance factors in addition to the *Rpi-blb1, Rpi-blb2* and *Rpi-blb3* genes

Analysis of the sequences obtained in different allele mining strategies suggests an evolution of the major late blight locus on linkage group IV through recombination and point mutations. The identification of the repeats and amino acids in the LRR domain which are specific for PiAVR2 recognition was possible by making use of the sequence information provided by the alleles.

Finally the results, described in this thesis, have been discussed in a potato/ *P. infestans* co-evolution context.

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SAMENVATTINGl

Aardappel wordt wereldwijd gegeten en is het op twee na belangrijkst voedingsgewas na rijst en tarwe. Aardappelrassen laten een grootte verscheidenheid aan kleur, vorm, smaak, kookeigenschappen en zetmeelgehalten zien, maar zijn allemaal afkomstig van dezelfde soort: *Solanum tuberosum*. Aardappelveredeling is voor internationale georiënteerde veredelingsbedrijven een economisch belangrijke activiteit, maar het speelt ook een belangrijke rol bij het doorbreken van de armoedecirkel waar kleine boeren zich in bevinden. In de Andes gebruiken boeren verschillende aardappelgenotypen gecombineerd met landbouwtechnieken die al duizenden jaren mondeling worden doorgegeven.

De belangrijkste bedreiging voor de aardappelproductie is de Aardappelziekte die wordt veroorzaakt door de oömyceet *Phytophthora infestans* die bladeren, stengels en knollen aantast en verwoest. Verschillen tussen de manier waarop aardappel wordt gecultiveerd in Zuid-Amerika en de rest van de wereld houdt verband met de verschillen in de consequenties van *Phytophthora* infecties. In de 19de eeuw werden hele aardappelvelden in Ierland verwoest terwijl in Zuid-Amerika de verspreiding van *P. infestans* gemakkelijk werd tegengehouden. Dit verschil wordt verklaard door de grote hoeveelheid biodiversiteit zoals die te vinden is in de Chiloé archipelago in Chili waar de plaatselijke bevolking ongeveer 200 verschillende variëteiten van de inheemse aardappel verbouwen. De genetische diversiteit van de gecultiveerde inheemse aardappel gedraagt zich als een schild tegen dit onberekenbare pathogeen. Geïnspireerd door dit model zouden landbouwers en veredelaars de genetische diversiteit in de Europese aardappel meer moeten gebruiken, om problemen te voorkomen die veroorzaakt worden door het te intensief gebruikt van aardappel als monocultuur.

Tijdens het verkennen van de Zuid-Amerikaanse inheemse aardappelcollectie, *bleken Solanum demissum* en later ook *Solanum bulbocastanum* een bron te zijn van resistentiegenen tegen *P. infestans (Rpi)*. De *S. demissum Rpi* genen werden overgebracht in aardappelveredelingsklonen door traditionele introgressie veredeling. Toch werd doorbreking van deze mogelijkheid, om effectieve resistentie tegen de Aardappelziekte te verschaffen, binnen een decennium waargenomen. Met als doel om een hoopvolle en meer duurzame bescherming in bestaande cultivars te verkrijgen, stelden wetenschappers in de plantenveredeling voor, om inheemse Zuid-Amerikaanse aardappel *Rpi* genen direct in moderne aardappelrassen te introduceren via de zogenaamde cisgenese aanpak. Hierbij worden resistentiegenen van aardappel zelf of van kruisbare wilde soorten gebruikt. Dit in tegenstelling tot de transgene aanpak, waarbij planten genen geïntroduceerd kunnen krijgen die afkomstig zijn van niet gerelateerde geslachten of zelfs andere rijken. Veredelen van cisgenese planten is goed op weg naar publieke

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acceptatie door de impliciete gelijkenis met natuurlijke kruisingen en omdat de principes van cisgenese vanuit de wetenschappelijke gemeenschap goed uitgelegd kunnen worden.

Door de flexibele eigenschap van *P. infestans*, om de bescherming van de nieuw geïntroduceerde *Rpi* genen te overwinnen, wordt nog steeds getracht nieuwe meer duurzame *Rpi* genen te ontdekken en te kloneren uit inheemse aardappelen. Hierdoor is er een nieuwe familie van *Rpi* genen gekarakteriseerd die vertegenwoordigd wordt door *Rpi-blb3*, *Rpi-abpt*, *R2, R2-like* en *Rpi-mcd1.1* die afkomstig zijn van *S. bulbocastanum*, *S. demissum, S. edinense* en *S. microdontum.* Tijdens dit onderzoek zijn we erin geslaagd deze genen fysiek te isoleren, de moleculaire karakterisatie van hun functionaliteit te bepalen en hun allelische verdeling in de Petota collectie in kaart te brengen.

Rpi-blb3, Rpi-abpt, *R2, R2-like* en *Rpi-mcd1.1* behoren tot de aardappel koppelingsgroep IV en hebben allemaal een specifieke sequentie die karakteristiek is voor LZ-NBS-LRR eiwitten. Het meest gelijkende R gen dat tot nu toe bekend is, is RPP13 van *Arabidopsis thaliana* die wat betreft aminozuur sequentie 35% ermee overeenkomt. De LRR domeinen van de Rpi-blb3, Rpi-abpt, R2 en R2-like eiwitten zijn zeer homoloog, terwijl de LZ en NBS domeinen het meest polymorf zijn waarbij die van R2 het meest divergent is. Alle vier de *Rpi* genen herkennen het recent geïdentificeerde RXLR effectoreiwit PiAVR2 welke door *P. infestans* in het cytoplasma van de plantencel wordt uitgescheiden tijdens het infectieproces. In tegenstelling tot *Rpi-blb3, Rpi-abpt, R2* en *R2-like*, gaat het *S. microdontum* resistentiegen *Rpi-mcd1.1* geen interactie aan met PiAVR2 en heeft een ander resistentiespectrum. *Rpi-mcd1.1* komt op DNA nucleotide niveau voor 90% overeen met *Rpi-blb3* en de polymorfe nucleotiden zijn vooral gelokaliseerd in de LRR regio.

De *S. bulbocastanum* haplotypen van *Rpi-blb1, Rpi-blb2* en *Rpi-blb3* werden ontdekt in een aantal Mexicaanse diploïde en polyploïde soorten die verwant aan *S. bulbocastanum* zijn. Deze drie resistentiegenen komen in verschillende combinaties en frequenties voor in *S. bulbocastanum* accessies en hun verspreiding is beperkt tot Centraal Amerika. Een geselecteerde set genotypen was getest op hun reactie op de avirulentie effectoren IPIO-2, Avr-blb2 en Pi-Avr2, welke een interactie aangaan met *Rpi-blb1*, *Rpi-blb2* en *Rpi-blb3*, respectievelijk. Deze set was ook getest na inoculatie met verschillende isolaten. Met deze aanpak konden enkele accessies geïdentificeerd worden die nieuwe, nog onbekende, *Phytopthora* resistentiefactoren bevatten naast de al bekende *Rpi-blb1, Rpi-blb2* en *Rpi-blb3* genen.

Analyse van de sequenties verkregen uit de verschillende “allele mining” strategieën suggereren het optreden van een evolutieproces van dit belangrijke *Phytopthora* resistentielocus, dat op recombinaties en puntmutaties gebaseerd is. Door gebruik te maken van de sequentie informatie van deze allelen

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konden repeats en aminozuren in het LRR domein geïdentificeerd worden die specifiek voor de PiAVR2 herkenning zijn.

In het laatste hoofdstuk worden de resultaten die in dit proefschrift beschreven zijn in de context van een co-evolutie van aardappel/ *P. infestans* besproken.

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RESUME

La pomme de terre joue un rôle clé dans le système alimentaire mondial. C'est la principale denrée alimentaire non céréalière du monde. Dans les pays développés, la consommation de pommes de terre augmente considérablement et représente plus de la moitié de la récolte mondiale. Comme elle est facile à cultiver et que sa teneur énergétique est élevée, c'est une culture commerciale précieuse pour des millions d'agriculteurs. Elle est vivement recommandée pour atteindre la sécurité alimentaire et elle peut aider les agriculteurs à faibles revenus et les consommateurs vulnérables à surmonter la crise actuelle des disponibilités alimentaires et de la demande mondiales.

La pomme de terre est consommée dans les Andes depuis 8 000 ans environ, les agriculteurs sont parvenus à sélectionner et à améliorer les premiers spécimens de ce qui allait donner, au fil des millénaires, une diversité inouïe de tubercules. Bien qu'offrant à ses consommateur un choix de couleurs, forme, saveur, propriétés de cuisson, de contenu en amidon etc..La pomme de terre que nous connaissons, l'espèce *Solanum tuberosum*, ne contient en réalité qu'une infime partie de la diversité génétique contenue dans les sept espèces reconnues et dans les 5 000 variétés de pommes de terre qui sont encore de nos jours cultivées dans les Andes.

Cette absence de diversité génétique chez les clones de tubercules cultivés en Amérique du Nord et en Europe a pour conséquence de les rendre extrêmement vulnérables: si un ravageur ou une maladie s'attaquait à une plante, il pouvait se propager rapidement aux autres. Un exemple fut le mildiou de la pomme de terre causé par l'oomycète *Phytophthora infestans*, qui dévasta les champs de pommes de terre d'Europe continentale, de la Belgique à la Russie. Mais le pays le plus affecté fut l'Irlande, où la pomme de terre représentait plus de 80 pour cent de la ration énergétique. Entre 1845 et 1848, le mildiou de la pomme de terre ravagea trois récoltes, provoquant une famine qui causa la mort d'un million de personnes.

De toute évidence, la diversité génétique de la pomme de terre ainsi que les méthodes agricoles pratiquée dans la cordières des Andes agissent comme un bouclier contre les différents bio agresseurs. Inspiré par ce modèle pour résoudre les problèmes soulevés par l'utilisation intensive de la monoculture de pommes de terre et des épidémies de mildiou, agriculteurs et ingénieurs agronomes pensent qu'il est nécessaire de maintenir la diversité génétique dans les cultures de pomme de terre, en mettant au point des variétés plus productives et résistantes aux maladies..

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En explorant le contenu génétique des pommes de terres indigènes collectionnées a l occasion de différentes expéditions en en Amérique du Sud dans les années 60, il fut découvert de nouvelles espèces contenant une variétés de gènes de résistances au mildiou (Rpi : resistance à *Phytophthora infestans*), qui sont *Solanum demissum* et, plus tard, *Solanum bulbocastanum* . Les gènes de résistance au mildiou présent dans le matériel génétique de *S. demissum*. ont été transmis aux nouvelle variétés de pomme de terre par sélection traditionnelle. L effet souhaité en champs fut de courte durée car la résistance aux mildiou apportée par les nouveaux gènes fut contournée en une décennie par une adaptation rapide de *P. infestans*.

Une autre solution pour rapidement fournir une protection totale, espérons-le, plus durable aux varietes existantes de pommes de terre est d'y introduire directement les gènes de résistances indigènes d'origine sud américaine en utilisant une approche dite cisgenic. Les plantes cisgeniques sont modifiees exclusivement par des gènes provenant du meme genre de plante, à l'inverse des plantes transgéniques qui elles, peuvent contenir des gènes qui provenant de genre non apparentés ou même du monde animal. L'acceptation des plantes cisgenic par le public est en bonne voie grâce aux efforts déployés par la communauté scientifique pour expliquer les principes de cisgenesis ainsi que du fait que ces plantes soient fortement comparable à celle obtenues par sélection traditionnelle.

Des leçons ont été tirées sur la flexibilité de P. infestans pour contourner l'effet de resistance procurer par les gènes nouvellement introduits et, par conséquent, les efforts sont maintenus pour en découvrir et en cloner de nouveaux. Dans cet esprit, une nouvelle famille de gènes de résistance aux mildiou représentée par *Rpi-blb3*, *Rpi-abpt*, *R2*, *R2-like* et *Rpi-mcd1.1* ont été caractérisés dans des clones issus des especes de pommes de terre *S.bulbocastanum* , *S.demissum*, *S.edinense* et *S.microdontum*. Nous avons accompli dans cette recherche l'isolement physique de ces gènes, la caractérisation moléculaire de leurs fonctionnalités ainsi que leur distribution allelic dans la collection de pommes de terre sud américaine dénommée « Petota ».

Les gènes *Rpi-blb3*, *Rpi-abpt*, *R2*, *R2-like* et *Rpi-mcd1.1* sont localisés sur le chromosome numéro IV de la pomme de terre et tous contiennent des séquences caractéristiques des protéines appartenant a la catégorie LZ-NBS-LRR (LZ : leucine zipper, NBS : nucleotide binding site, LRR : leucine rich repeat). Le gène de résistance le plus similaire est *RPP13* provenant de la plante modèle *Arabidopsis thaliana*, avec un taux de similitude des séquence protéiques de 35 %. Les domaines LRR des genes *Rpi-blb3*, *Rpi-abpt*, *R2* et *R2-like* sont très homologues, tandis que les domaines LZ et BNS sont plus polymorphes avec ceux de R2, ce dernier étant le plus divergent. Les protéines issue des quatre gènes reconnaissent la protéine de type RXLR récemment identifiée, PiAVR2, qui est sécrétée par *P.infestans* dans le cytoplasme des cellules végétales au cours du processus d'infection. Contrairement à *Rpi-blb3*,

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Rpi-abpt, *R2* et *R2-like*, la proteine issue du gène de résistance provenant de *S.microdontum*, *Rpi-mcd1.1,* n'interagit pas avec PiAVR2 et fournit un spectre de résistance différent. *Rpi-mcd1.1* est similaire à 90 % au niveau nucléotidique à *Rpi-blb3* et les nucléotides polymorphes sont principalement situées dans la région LRR.

Les haplotypes *Rpi-blb1*, *Rpi-blb2* et *Rpi-blb3* à l'origine identifiés dans l'èspèce *S. bulbocastanum*, ont été découverts dans plusieurs espèces mexicaines diploïdes et polyploïdes étroitement apparentées à *S. bulbocastanum*. Différentes combinaisons des trois gènes de résistance ont été trouvées et leur distribution est limitée à l'Amérique centrale. Un jeu de génotypes a été sélectionné et testé pour leur réponse aux protéines secretees par *P. infestans*, IPIO-2, Avr-blb2 et PI-Avr2, qui interagissent avec Rpi-blb1, Rpi-blb2 et Rpi-blb3, respectivement. Leurs niveaux de résistance à différent isolats de *P. infestans* ontaussi été évalués. Cette approche as permis d' identifier de nouvelles especes pouvant contenir des facteurs de résistance au mildiou encore inconnus, en plus de *Rpi-blb1*, *Rpi-blb2* et *Rpi-blb3*.

L'analyse des séquences obtenues à partir des différentes stratégies d'« allele mining » suggère une évolution du locus de résistance au mildiou localisé sur le chromosome IV, par recombinaison et mutations ponctuelles. En explorant les différences existant entre les différent allèles, nous avons identifié quelles parties du domaine LRR, sont importantes dans la reconnaissance de PiAVR2.

Enfin, les résultats décrits dans cette thèse ont été discuté dans un contexte de co-évolution entre la pomme de terre et *P. infestans*.

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When I started my PhD, scientific research was the core of my life, working late in the nights and weekends. And I met **Teun**, my angel on earth, who makes me realize that the sky was blue, birds were singing, flowers were colorful and beautiful. It was in spring 2006. We got married, two times, in Togo and in the Netherlands….nine months later **Maelya** was born. It was the 1st of July 2009. Teun, thank you for this wonderful gift you gave me, your LOVE;

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Thank you for your patience, your tolerance, your energy, your support in this life. **Teun** and **Maelya**, you are the core of my life. I love you.

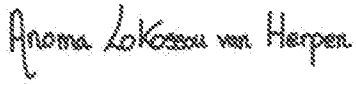
Ria, **Riny**, **Jan**, **Nelly**, **Maarten**, **Melina**, **Wendy**, **Nathalie**, **Iris** en **Mike,** dank jullie wel**.** Jullie hebben jullie armen en hart geopend en me warm en onvoorwaardelijk ontvangen in jullie familie.

This acknowledgment part wouldn't be complete without a special part to **those who left**.

I first would like to thank you **Bud** for your humor and positive attitude in all circumstances, until the end. Rest in peace.

Thank you to the anonymous **donator** who made a legacy gift to Wageningen University fund and permitted to finance my project. I hope that I fulfill his/her wish.

To finish, Thank you to my **ancestors**, **family** and **friends** who kept an eye on me and guide me on the good tracks through this life. M' do Akpe ne mawu. Akpe ne mia togbe o. Agnigban ne fa na mi.



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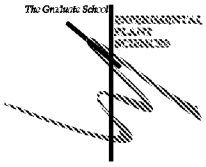


CURRICULUM VITAE

Anoma Akuvi Lokossou was born on September the 7th, 1978 in Nice located on the sunny Mediterranean coast of France. In 1981, she moved with her family to Lomé, the capital of Togo. After she completed her high academic school at the "Lycée français de Lomé" in 1996, she returned to Nice to study biology at the University of Sofia Antipolis. In 2002, she continued with plant biotechnology and bioingeniery study at the University of Paul Sabatier in Toulouse (France). In February 2005, Prof. Dr. Richard Visser gave her the opportunity to do a 6 months internship in Wageningen University at the Laboratory of Plant Breeding (The Netherlands). She worked in the team of Dr. Krit Raemakers on improving the in vitro genetic transformation of Cassava and analyzing the protein content of transgenic pea plants. After she obtained her master degree in September 2005, she started her PhD in Plant breeding under the supervision of Prof. Dr. Richard Visser, Prof. Dr. Ir. Evert Jacobsen and Dr. Edwin van der Vossen. The results of her research are described in this thesis.

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Education Statement of the Graduate School
Experimental Plant Sciences



Issued to: Anoma A. Lokossou
Date: 7 June 2010
Group: Laboratory of Plant Breeding, Wageningen University

1) Start-up phase	*date*
► **First presentation of your project**	
Late blight resistance genes: Rpi-blb3, Rpi- abpt, R2-like	Mar 20, 2006
► **Writing or rewriting a project proposal**	
► **Writing a review or book chapter**	
► **MSc courses**	
Genetic Analysis, Tools and Concepts	Oct 31-Nov 19, 2005
► **Laboratory use of isotopes**	
Radiation expert level 5B	Mar 19, 2007
Subtotal Start-up Phase	*9.0 credits**

2) Scientific Exposure	*date*
► **EPS PhD student days**	
EPS PhD Student day, Wageningen University	Sep 19, 2006
EPS PhD Student day, Wageningen University	Sep 13, 2007
EPS PhD Student day, Leiden University	Feb 26, 2009
► **EPS theme symposia**	
EPS theme 4 symposium 'Genome Plasticity', Wageningen University	Feb 09, 2005
EPS theme 2 symposium 'Interactions between Plants and Biotic Agents', University of Amsterdam	Feb 02, 2007
► **NWO Lunteren days and other National Platforms**	
NWO-ALW meeting, Lunteren	Apr 02-03,2007
NWO-ALW meeting, Lunteren	Apr 07-08, 2008
NWO-ALW meeting, Lunteren	Apr 08-09, 2009
► **Seminars (series), workshops and symposia**	
Seminar Sophien Kamoun	Oct 05, 2005
Seminar Tony Conner at Keygene	Jun 29, 2005
Seminar Dr. Kurt H. Lamour: "Phytophthora capsici: the key to unlocking Oomycete genetics and host specificity".	Nov 06, 2006
Seminar Dr. Roger Hellens: Identification and functional analysis of transcription factor genes from apple and their role in fruit quality-related traits	Feb 14, 2007
Research day (Plant breeding)	2007
Research day (Plant breeding)	Mar 2009
► **Seminar plus**	
► **International symposia and congresses**	
Oomycete Molecular Genetics Network Wageningen	May 04-07, 2006
MPMI (Italy: Sorrento)	Jul 21 -27, 2007
Sol meeting (Koln)	Oct 12-16, 2008
► Bio-exploit meeting	Feb 25-27, 2009
► **Presentations**	
Poster presentation at Oomycete Molecular Genetics Network Wageningen	May 04-07, 2006
Poster presentation at lunteren	Apr 02-03,2007
Poster presentation at Sorento	Jul 21 -27, 2007
Oral presentation at lunteren	Apr 07-08, 2008
Poster presentation at 5th Solenaceae genome workshop	Oct 12-16, 2008
► **IAB interview**	Sep 13, 2007
► **Excursions**	
Avebe excursion	Sep 2006
Subtotal Scientific Exposure	*13.6 credits**

3) In-Depth Studies	*date*
► **EPS courses or other PhD courses**	
Signaling in plant development	Jun 19-21, 2006
Gateway to gateway technology	Nov 20-24, 2006
International PhD summerschool "Environmental signaling: Arabidopsis as a model"	Aug 27-29, 2007
► **Journal club**	
► Literature discussion "Plant Breeding"	2005-2009
► **Individual research training**	
Subtotal In-Depth Studies	*6.0 credits**

4) Personal development	*date*
► **Skill training courses**	
6th Frans-Nederlandse Ontmoetingsdagen: Promoveren zonder grenzen (Paris)	May 22-24, 2006
PhD competence assessment	Jun 02, 2006
Teaching and supervising Thesis students	Jun 13-14, 2006
Personal Efficacy	Sep 27-Oct 25, 2006
Toefle test (personal training)	Apr 26, 2008
Course Career perspectives	Oct 20-Nov 24, 2009
► **Organisation of PhD students day, course or conference**	
Lab trip of Plant breeding laboratory	Jun 2006
► **Membership of Board, Committee or PhD council**	
Subtotal Personal Development	*6.6 credits**

TOTAL NUMBER OF CREDIT POINTS*	**35.2**

Herewith the Graduate School declares that the PhD candidate has complied with the educational requirements set by the Educational Committee of EPS which comprises of a minimum total of 30 ECTS credits

** A credit represents a normative study load of 28 hours of study*

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Cover Art / Photo CV / Invitation:

Remko Zijlstra 2010

www.RemkoZijlstra.vpweb.nl

Ezel1@live.nl

Remko Zijlstra created this cover inspired by the history of the potato combined with reading this thesis summary.

The cover depicts the journey of the potato across time, presented by a serie of maps, from the front to the back, as a "come back" to the origin.

This project was financed by a legacy to the Wageningen University Funds (WUF)

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e-Xtra*

Exploiting Knowledge of *R/Avr* Genes to Rapidly Clone a New LZ-NBS-LRR Family of Late Blight Resistance Genes from Potato Linkage Group IV

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In addition to the resistance to *Phytophthora infestans* (*Rpi*) genes *Rpi-blb1* and *Rpi-blb2*, *Solanum bulbocastanum* appears to harbor *Rpi-blb3* located at a major late blight resistance locus on LG IV, which also harbors *Rpi-abpt*, *R2*, *R2-like*, and *Rpi-mcd1* in other *Solanum* spp. Here, we report the cloning and functional analyses of four *Rpi* genes, using a map-based cloning approach, allele-mining strategy, Gateway technology, and transient complementation assays in *Nicotiana benthamiana*. *Rpi-blb3*, *Rpi-abpt*, *R2*, and *R2-like* contain all signature sequences characteristic of leucine zipper nucleotide binding site leucine-rich repeat (LZ-NBS-LRR) proteins, and share amino-acid sequences 34.9% similar to RPP13 from *Arabidopsis thaliana*. The LRR domains of all four Rpi proteins are highly homologous whereas LZ and NBS domains are more polymorphic, those of R2 being the most divergent. Clear blocks of sequence affiliation between the four functional resistance proteins and those encoded by additional *Rpi-blb3* gene homologs suggest exchange of LZ, NBS, and LRR domains, underlining the modular nature of these proteins. All four *Rpi* genes recognize the recently identified RXLR effector PiAVR2.

The potato (*Solanum tuberosum*) ranks as the world's third most important food crop after wheat and rice. Compared with these other staple crops, a greater proportion of the potato crop is edible and a higher yield per hectare is achieved, which, combined with its high nutritional value, underlines its importance and explains ongoing increases in global potato production. The most significant threat to potato production worldwide is the late blight disease, caused by the oomycete pathogen *Phytophthora infestans*, which destroys leaves, stems, and tubers. Current disease control relies on multiple (up to 20) fungicide applications per season (Hanson et al. 2007) and costs associated with chemical control amount to €5 billion globally per year (Whisson et al. 2001). Such pesticide inputs are both environmentally and economically undesirable and have a negative impact on the sustainability of potato production. Host resistance is a more environmentally benign means of restricting late blight infection but the success of this management practices hinges on effective and durable host resistance and an understanding of the pathogen population.

Early in the 20th century, the potential of the wild Mexican hexaploid species *S. demissum* was recognized for conferring extreme levels of late blight resistance, and hybrids with *S. tuberosum* were generated. Subsequently, 11 major dominant resistance (*R*) genes were identified and introgressed into potato cultivars (Malcolmson 1969; Malcolmson and Black 1966; Müller and Black 1952). However, deployment of these single genes did not provide durable resistance due to the rapidly changing populations of *P. infestans*. As an alternative approach, breeders started to select for quantitative "field" resistance using races of *P. infestans* that were compatible with the *R* genes present in their breeding material (Black 1970; Toxopeus 1964). Despite considerable progress in genetic analysis of quantitative resistance to late blight using molecular markers (Gebhardt and Valkonen 2001; Simko 2002), under long day conditions, breeders have achieved little progress, the major drawbacks being tetrasomic inheritance of potato and the strong linkage between foliage resistance and late foliage maturity (Bradshaw et al. 2004; Oberhagemann et al. 1999; Visker et al. 2003). Therefore, it is anticipated that breeding for late blight resistance in potato, aimed at substantially contributing to disease management, requires the deployment of major resistance to *P. infestans* (*Rpi*) genes. Recent germplasm screens are revealing a wealth of *Rpi* genes in wild *Solanum* spp. and the challenge now is how to select, judiciously combine, and deploy sets of *Rpi* genes that can confer durable late blight resistance in modern potato cultivars.

Key to durable exploitation of *Rpi* genes is also a better understanding of their interactions with effectors from *P. infestans*. All microbes trigger immune responses in plants via host receptor-mediated recognition of pathogen-associated molecular patterns (PAMPs). Such PAMP-triggered immunity (PTI) can be suppressed by secretion of effectors which act either outside

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Current address of E. A. G. van der Vossen: Keygene N.V., Agro Business Park 90, 6708 PW Wageningen, The Netherlands.

*The e-Xtra logo stands for "electronic extra" and indicates that three supplementary tables and three supplementary figures are published online.

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or inside host cells and reprogram host metabolism to promote effector-triggered susceptibility (ETS). When a successful pathogen has suppressed PTI, the products of plant *R* genes, as a second "layer" of defense, can directly or indirectly detect effectors (termed avirulence [AVR] proteins). R proteins subsequently activate resistance responses (effector-triggered immunity [ETI]), often including programmed cell death during the hypersensitive response (HR) (Jones and Dangl 2006). The ability of a pathogen to evade recognition in the *R*-gene-for-*Avr*-gene concept (Flor 1971) can be explained by an evolutionary "arms race" (Bergelson et al. 2001; Holub 2001). The generation, through mutation, and selection of *AVR* alleles that evade recognition is matched by strong selection pressure for *R* gene alleles that retain recognition. This is particularly evident in the ATR1-RPP1 and ATR13-RPP13 interaction in the *Hyaloperonospora arabidopsidis* (formerly *H. parasitica*)–*Arabidopsis* pathosystem, where considerable allelic diversity has been revealed by studying natural host and pathogen populations (Allen et al. 2008; Rehmany et al. 2005). In naturally co-evolving *P. infestans*–potato populations, similar high levels of allelic diversity are expected to be observed. So far, studies of the PiAVR3a–R3a interaction reveal diversifying selection for both genes (Armstrong et al. 2005; Huang et al. 2005). Recently, several oomycete *AVR* genes were identified (Allen et al. 2004; Armstrong et al. 2005; Rehmany et al. 2005; Shan et al. 2004; Vleeshouwers et al. 2008). Although each of the AVR proteins is distinct at the level of primary sequence similarity, they all share a secretion signal peptide followed by the motif RXLR and an acidic region, often ending in the sequence EER (Birch et al. 2008a and b; Whisson et al. 2007). The RXLR-EER region is required for translocation of PiAVR3a, following its secretion from *P. infestans* haustoria, to the inside of host plant cells (Whisson et al. 2007). Translocation of RXLR-containing proteins inside the plant cell is consistent with their recognition by host nucleotide binding site leucine-rich repeat (NBS-LRR) resistance proteins (Birch et al. 2006). Because all oomycete AVR proteins identified to date have the RXLR motif, it provides a signature to identify AVR candidates from the *P. infestans* genome. Future knowledge of RXLR effectors diversity and their survey in *P. infestans* population could predict the lifespan of *Rpi* genes in specific areas.

Conventional introgression of resistance often involves interspecific crosses and repeated backcrosses with cultivated potato, which is a very slow and inefficient process. Currently, *Rpi* genes of natural origin, so-called cisgenes (Jacobsen and Schouten 2007; Schouten and Jacobsen 2008), can be introduced using marker-free transformation systems leading to cisgenic plants with only the genes of interest and without linkage drag of other genes from the wild species or from antibiotic resistance genes normally present at the T-DNA (de Vetten et al. 2003; Schaart et al. 2004; Yu et al. 2006). To date, six *Rpi* genes have been reported: *R1* and *R3a* from *S. demissum* (Ballvora et al. 2002; Huang et al. 2005), *RB/Rpi-blb1* and *Rpi-blb2* from *S. bulbocastanum* (Song et al. 2003; van der Vossen et al. 2003, 2005), and *Rpi-sto1* and *Rpi-pta1* from *S. stoloniferum* and *S. papita*, respectively (Vleeshouwers et al. 2008). All belong to the NBS-LRR class of plant R proteins, which are thought to be cytoplasmic (van der Biezen and Jones 1998). Various loci are reported to contain *Rpi* genes on linkage group (LG) VII (Kuhl and Hanneman 2001), LG IX (Sliwka et al. 2006; Smilde and Brigneti 2005), LG X (Ewing and Simko 2000; Rauscher et al. 2006), and LG XI (Bradshaw et al. 2006; Huang et al. 2004, 2005).

Here, we describe the cloning and functional characterization of *Rpi-blb3*, *Rpi-abpt*, *R2*, and *R2-like* from the major late blight resistance locus on LG IV of potato (Li et al. 1998; Park et al. 2005a, b, and c). Upon map-based cloning of *Rpi-blb3*, an efficient candidate gene allele-mining approach in combination with transient complementation in the heterologous *Nicotiana benthamiana* "model" system led to the rapid cloning of the other three genes. All four *Rpi* genes code for proteins of the NBS-LRR class of R proteins. Interestingly, with respect to known functional R proteins, they share the highest similarity (34.9% amino acids) with RPP13 from *Arabidopsis thaliana* (Bittner-Eddy et al. 2000), which confers resistance to the oomycete *H. arabidopsidis*. Despite apparent differences between the four functional R proteins, all four induce an HR upon coexpression with the *P. infestans*-derived RxLR effector PiAVR2 (E. Gilroy, *personal communication*) in *N. benthamiana* leaves, indicating that they recognize the same effector protein.

RESULTS

Cloning of *Rpi-blb3* and *Rpi-abpt*.

In order to clone *Rpi-blb3* and *Rpi-abpt*, two bacterial artificial chromosome (BAC) libraries were constructed using DNA derived from the resistant clones Blb99-256-3 and 707TG11-1, respectively. Approximately 74,000 clones with an average insert size of 85 kb, corresponding to eight genome equivalents, were obtained for each library. These libraries were screened initially with sequence-characterized amplified region marker Th21, which cosegregated with resistance in mapping populations of 1,396 and 1,383 F1 progeny plants (Park et al. 2005a). In this way, BAC clones Blb22 and TG9, respectively, were identified (Fig. 1A and 1B). By sequencing the ends of these two BACs, new markers were developed which were used to define the genetic intervals of the *R* loci more precisely and to rescreen the BAC libraries to identify clones that overlapped with the initial BAC clones. In this way, the *Rpi-blb3* locus was delimited to a 0.1-centimorgan (cM) interval (EAAC/MAAC_99- Blb25-T; 2/1,396 recombinants) that is physically spanned by the two partially overlapping BAC clones Blb22 and Blb25 (Fig. 1A). In the case of the *Rpi-abpt* locus, the partially overlapping BAC clones TG9 and TG77 were identified. One end of the contig cosegregated with resistance (TG77-S) while the other mapped 0.1 cM proximal to *Rpi-abpt* (TG9-S) (Fig. 1B).

To gain insight into the molecular structure of the *R* loci under study, BAC clones Blb22 and TG9 were sequenced to 6x coverage. This revealed that clone Blb22 did not contain any *R* gene homologues (RGH) whereas clone TG9 contained two RGH, which shared significant similarity to RGH present in the sequenced tomato BAC clone AF411807L (Van der Hoeven et al. 2002) (Fig. 1). BAC clones Blb25 and TG77 were subsequently screened for the presence of RGH-specific sequences through polymerase chain reaction (PCR) analysis using the primers 4-PLOOP-F and 4-GLPL-R (Table 1), which were designed by aligning the RGH sequences of clone AF411807L with those present on BAC clone TG9. Southern analysis of BAC clones Blb22, Blb25, TG9, and TG77 using an RGH-specific PCR fragment amplified from BAC clone Blb25 as a probe identified a minimum of two RGH on BAC Blb25 and TG9 and one RGH on TG77 located on the overlapping part with TG9 (Fig. 1).

Libraries harboring random overlapping binary subclones of 8 to 10 kb were generated from BAC clones Blb25 and TG9. In total, 1,152 clones per library were screened for the presence of RGH using primers 4-GLO2-F and 4-GLO2-R (Table 1). Based on restriction analyses of the PCR fragments, RGH-positive subclones were divided into separate classes: *Blb3GH-A* and *Blb3GH-B* for *Rpi-blb3* and *AbptGH-A* and *AbptGH-B* for *Rpi-abpt*. After determining the relative positions of the RGH within the 8- to 13-kb subclones, candidates from each class were tar-

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geted for transformation to the susceptible potato cv. Desiree. Transformation experiments carried out with subclones harboring candidates *Blb3GH-A*, *Blb3GH-B*, *AbptGH-A*, and *AbptGH-B* resulted in numerous primary transformants. Detached leaf assays using isolates IPO-0 and 90128 revealed that all of the plants transformed with subclones harboring candidates *Blb3GH-A*, *AbptGH-A*, and *AbptGH-B* were susceptible to both isolates but that the majority (seven of eight) of the tested transgenic plants harboring *Blb3GH-B* reacted to both isolates with an HR (Fig. 2). In view of the differential response between the primary transformants harboring *Blb3GH-A* and *Blb3GH-B*, we designated *Blb3GH-B* as the *Rpi-blb3* gene.

In order to identify additional candidate genes for *Rpi-abpt*, the *Rpi-abpt*-specific BAC library was screened with TG77-S, leading to the identification of the TG77 overlapping BAC clone TG92 (Fig. 1B). Screening of this BAC clone with different sets of primers designed to amplify *AbptGH-A*, *AbptGH-B*, *Blb3GH-A*, or *Rpi-blb3* resulted in the identification of a third *Rpi-abpt* candidate gene, *AbptGH-C* (Fig. 1B). The *AbptGH-C* LRR domain was amplified, cloned, and sequenced using the primers Blb3-LRR-1-8 (Table 1). Sequence comparison of the three *abpt-GHs* enabled us to design an *AbptGH-C*-specific marker (Fig. 1B) which cosegregated with resistance. Southern blot analysis using the AbptGH-C ampli-

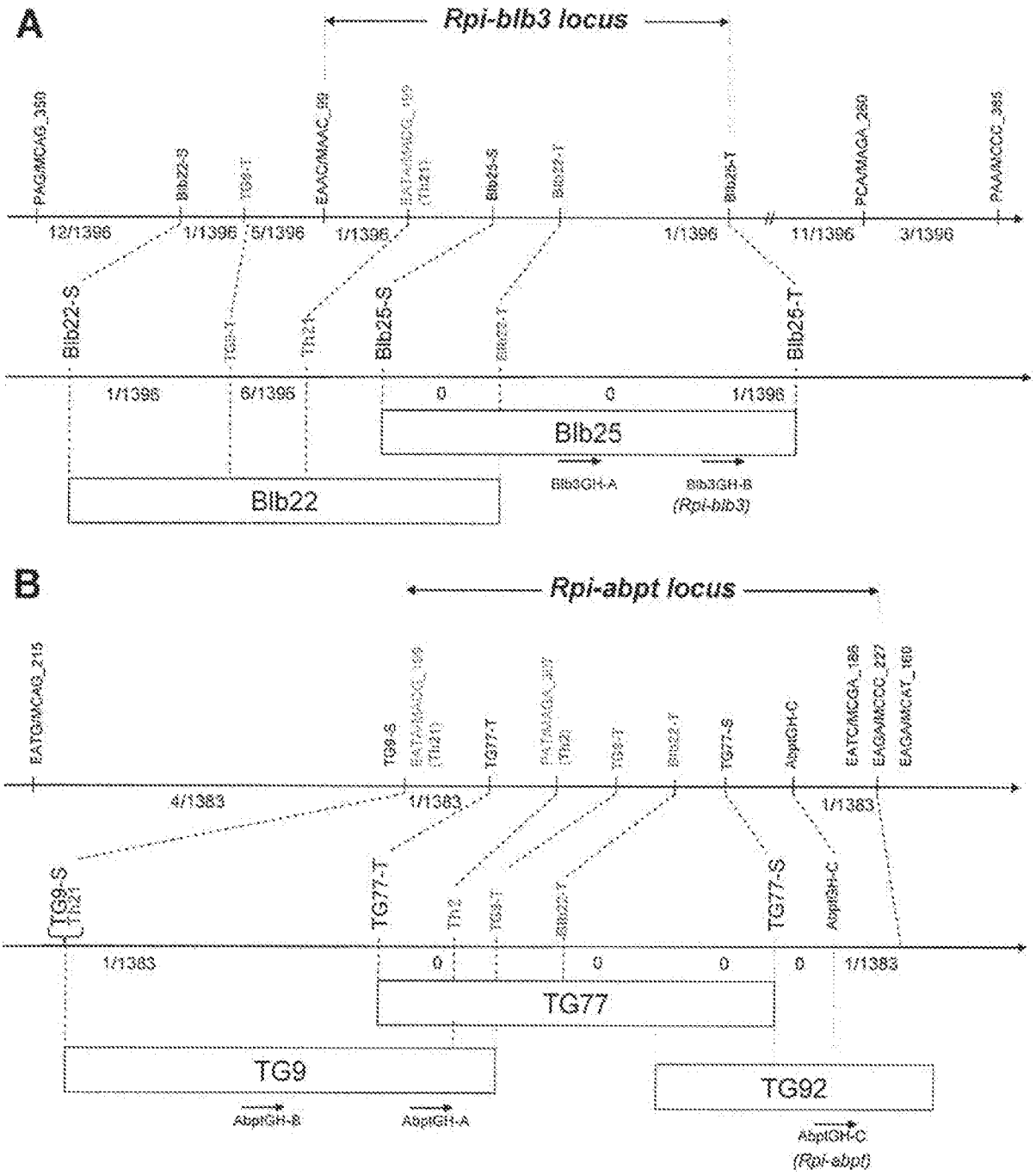


Fig. 1. Genetic and physical map of the **A**, *Rpi-blb3* and **B**, *Rpi-abpt* loci. Indicated are the relative positions of markers (in green are the one used in the mapping of both genes), the number of recombinants identified between markers, the overlapping BAC clones defining the two loci, and the relative positions of the resistance (*R*) gene candidates that have been targeted for complementation analysis.

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con as a probe revealed that clone TG92 contained one resistance gene encompassing an LRR domain highly similar to the one of *AbptGH-C*. Primers designed on the start and stop codon of *Rpi-blb3* were subsequently used to amplify a full-length AbptGH-C amplicon from clone TG92, which was cloned into the Gateway entry vector pDONR221. Using Multisite Gateway technology, the *AbptGH-C* amplicon was subsequently cloned into the binary pKGW-MG destination vector between *Rpi-blb3*-derived promoter and terminator sequences of 2,723 and 883 nucleotides, which were cloned into pDONR P4-P1R and pDONR P2R-P3 (Supplementary Fig. S1).

Complementation analysis was carried out in *N. benthamiana* using the *Agrobacterium tumefaciens* transient assay, whereby leaves from 4-week-old wild-type *N. benthamiana* plants were infiltrated with the *Agrobacterium* COR308 containing pKGW-AbptGH-C. The binary clones pBP-Rpi-blb3 and pKGW-Rpi-blb3, comprising the genomic *Rpi-blb3* gene and a Multisite Gateway reconstituted *Rpi-blb3* gene construct, respectively, were used as positive controls and pBP-AbptGH-A as a negative control. After 2 days, infiltrated leaves were inoculated with *P. infestans* strains PY23, IPO-0, and IPO-C in detached-leaf assays. Leaves infiltrated with pKGW-AbptGH-C, pBP-Rpi-blb3, and pKGW-Rpi-blb3 developed HR at the inoculation sites whereas noninfiltrated leaves and those infiltrated with pBP-AbptGH-A were susceptible to isolate PY23 and IPO-0. As expected, all leaves inoculated with IPO-C were susceptible (Fig. 3; Table 2). In view of these results, *AbptGH-C* was designated *Rpi-abpt*.

Allele mining for *R2* and *R2-like*.

Rpi-blb3 and *Rpi-abpt* belong to the major late blight resistance locus on LG IV that also harbors *R2* and *R2-like* (Li et al. 1998; Park et al. 2005c). In view of the conserved marker order and observed allelic conservation between the genetic maps of *Rpi-blb3*, *Rpi-abpt*, *R2*, and *R2-like* (Park et al. 2005c) and the high sequence conservation between *Rpi-blb3* and *Rpi-abpt*, we hypothesized that *R2* and *R2-like* were orthologues of *Rpi-blb3* and, thus, attempted to clone them through an allele-mining strategy. The same primers used to amplify the *Rpi-abpt* candidate gene from BAC clone TG92 were used to amplify full-length Blb3GH from the resistant parental genotypes Cebeco44158-4 (MaR2) and AM3778-16 harboring *R2* and *R2-like*, respectively. Amplicons of the expected size were cloned into pDONR221 and fully sequenced. In total, 8 unique sequences were obtained from AM3778-16 (R2-likeGH) and 19 from Cebeco44158-4 (R2GH), with amino acid identities between Rpi-blb3 and the novel Blb3GH ranging from 86.4 to 97.3% for R2-likeGH and 83.8 to 94.2% for R2GH (Supplementary Tables S1 and S2). Phylogenetic analysis of all the available amino acid sequences clustered one R2-like-GH and five R2GH in a clade together with the functional proteins Rpi-blb3 and Rpi-abpt (Fig. 4). The amino acid sequence of R2-likeGH-8 shares 97.3% amino acid identity with Rpi-blb3. R2GH-2, R2GH-8, R2GH-G3, R2GH-D3, and R2GH-65 share 94.2, 91, 92.6, 89.7, and 92.8% amino acid identity, respectively, with Rpi-blb3. This set of candidate genes was targeted for functional analysis and, therefore, cloned into the binary vector pKGW-MG between the *Rpi-blb3* promoter and terminator sequences, as described above for the *Rpi-abpt* gene.

Transient complementation assays in *N. benthamiana* indicated that R2GH-G3 and R2-likeGH-8 conferred resistance to Pi isolate IPO-0, whereas R2GH-2, R2GH-8, R2GH-D3, and R2GH-65 were nonfunctional (Fig. 3). Therefore, R2GH-G3 and R2-likeGH-8 were designated *R2* and *R2-like*, respectively.

Table 1. Overview of markers and primers used for mapping and cloning of *Rpi-blb3*

Name	Type	Polymerase chain reaction primer (5′ to 3′)	Tm[a]	Enzyme[b]
Th21	SCAR	F: ATTCAAAATTCTAGTTCCGCC	…	a.s.
	…	R: AACGGCAAAAAAGCACCAC	…	…
Blb22-S	CAPS	F: GTTTGATGTATGTTTGTTCTTGC	56	MspI
	…	R: TAATGCACTAATAACTAACTAGG	…	…
Blb22-T	SCAR	F: CTTTATTAGTTCCAAGAGCTAC	56	…
	…	R: ACCCATCCCTTTTTCCTTATC	…	…
Blb25-S	CAPS	F: ACAGATGCTACGTCCATCAC	56	AluI
	…	R: CTCCACATGCGATGCAAAAAG	…	…
Blb25-T	CAPS	F: TTTCGATTATGGTGAGCCTTC	56	Hpy 188
	…	R: TAGAAAAAGGGTGGTTGTGAC	…	…
RGH1	CAPS	F: GGSAAGACCACTCTTGCAAG	50	HpyCH4IV
	…	R: GGTTTTTAAGCTGCTAATGTTG	…	…
RGH2	SCAR	F: GGSAAGACCACTCTTGCAAG	50	a.s.
	…	R: TGGTYATAATYACTCTGCTGC	…	…
RGH3	CAPS	F: ATGRCTGATGCMTTTRTGTC	50	HaeIII
	…	R: CCYAAGTASAGAAAACACTGC	…	…
4-PLOOP	…	F: GGIATGGGIGGIYTIGGIARGAC	68	…
4-GLPL	…	R: TAGACAATGCAAGGGTAAMCC	…	…
4-GLO2	…	F: GTGTCTCTCAAGAGTACAACAC	56	…
	…	R: GCTCGAACATCAAGTAGTTTCC	…	…
Blb3-start	…	F: ATGGCTGATGCCTTTCTRTCATTTG	55	…
Blb3-end	…	R: TCAGGAATCTCCTTTAAATTTGGAC	…	…
Blb3-prom	…	F: TCTTCCTTAGCATTCGTAGC	55	…
	…	R: CTTTAGGAATACTAGTTTTGATTG	…	…
Blb3-int	…	F: AGCTTTTCTGCCAAGCACATTGG	55	…
	…	R: GTACCCTCCGTTTGTCGTTTGATC	…	…
Blb3-LRR-1-8	…	F: CTCTTTATGTATCAGACATGGC	55	…
	…	R: CAACATCTTTCCACTGATCAC	…	…
Blb3-prom -end	…	F: CCCCAAGTTGTATAATGGTTG	55	…
Blb3-orf-bg	…	R: TGCTTGAGTGATTGAATCTCC	…	…
Sto-orf-bg	…	R: GGCCATATTCAGACTGGGAG	…	…
Blb3-spe	…	F: AGCTTTTTGAGTGTGTAATTGG	55	…
	…	R: GTAACTACGGACTCGAGGG	…	…

[a] Annealing temperature. In AF411807R, annealing temperature was 48°C for the first seven cycles and 54°C for the last 30 cycles.

[b] Restriction enzymes that reveal polymorphism between resistant and susceptible linked alleles of the marker; a.s., allele-specific marker showing polymorphism without digestion.

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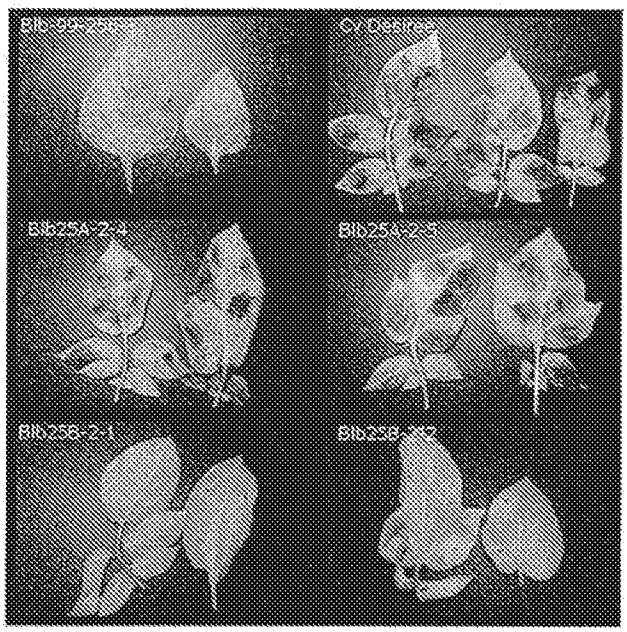


Fig. 2. Genetic complementation for late blight resistance. Eight days after inoculation with *Phytophthora infestans* isolate 90128, large necrotic lesions and massive sporulation were noted for potato cv. Desiree, and derived transformant Bib25A-2-4 and Bib25A-2-5, both expressing resistance gene homologue RGH-Bib25A. Typical resistance phenotype (i.e., hypersensitive response) was observed for Bib25B-2-1 and Bib25B-2-2 expressing RGH-Bib25B (*Rpi-blb3*).

All four *R* genes interact with the RXLR-EER effector *PiAVR2*.

Using a map-based cloning approach, markers flanking the *PiAVR2* locus (van der Lee et al. 1997) were used to delimit a physical region of 742,635 bp of the *P. infestans* genome sequence spanning the genetic interval containing *PiAVR2*. Within this region, two candidate *PiAVR2* genes on supercontig 16 of the *P. infestans* genome (16_7987 and 16_8044), each encoding a secreted RXLR-EER effector, were identified. The cloning and analyses of these gene candidates will be reported in detail in a separate publication (E. Gilroy, *personal communication*).

To functionally analyze the two RxLR-EER candidate genes identified at the *PiAVR2* locus in this study, *N. benthamiana* leaves were co-infiltrated with *A. tumefaciens* strains carrying a binary construct expressing one of the four *Rpi* genes under study together with strains carrying a binary construct expressing each of the two candidate RxLR-EER genes. Co-infiltration of *Rpi-blb3*, *Rpi-abpt*, *R2*, and *R2-like* with *16_7987* resulted in a hypersensitive cell death response (Fig. 5), whereas co-expression of these *Rpi* genes with the *16_8044* candidate or *PiAVR3a* failed to elicit visible cell death (Fig. 5) (E. Gilroy, *personal communication*). Based on these results, *16_7987* was designated *PiAVR2* and, clearly, all four *Rpi* genes under study interact with *PiAVR2*.

Gene structure and functionality.

Rpi-blb3, *Rpi-abpt*, *R2*, and *R2-like* are intronless genes which encode open reading frames (ORF) of 2,538 to 2,544 nucleotides that code for proteins of 845 to 847 amino acids harboring all the signature sequences characteristic of leucine

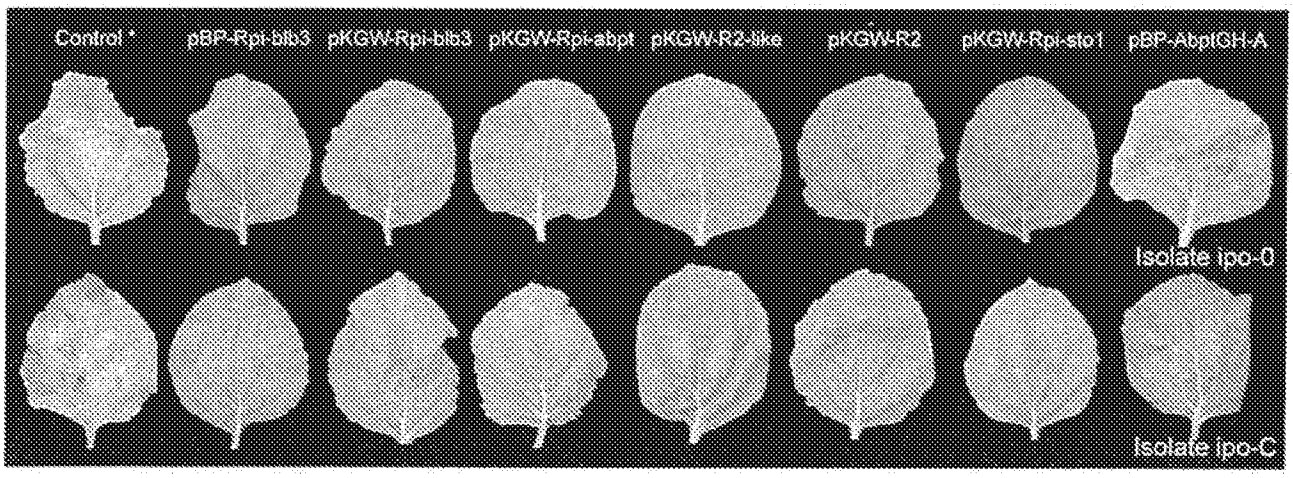


Fig. 3. Complementation of *Rpi-blb3*, *Rpi-abpt* , *R2*, *R2-like*, and *Rpi-sto1* by transient expression in *Nicotiana benthamiana*, followed by inoculation by *Phytophthora infestans* isolate Ipo0 (avirulent) and Ipo-C (virulent on all except *Rpi-sto1*). The prefix pBP stand for pBINplus, and pKGW stand for the gateway destination vector containing *Rpi-blb3* regulatory elements. In leaves expressing pB-Rpi-blb3, pKGW-Rpi-blb3, pKGW-Rpi-abpt, pKGW-R2-like, and pKGW-R2, a hypersensitive response was observed at 7 days postinoculation with *P. infestans* isolate Ipo-0, whereas large necrotic lesions and massive sporulation were evident for *P. infestans* isolate Ipo-C. Wild-type *N. benthamiana* (Control*) and the nonfunctional AbpIGH-A (pBP-AbpIGH-A) were included as susceptible controls, and Rpi-sto1 (pKGW-Rpi-sto1) was included as a resistant control.

Table 2. Overview of resistance screening with a set of 16 *Phytophthora infestans* isolates[a]

Species	Genotype	Gene	Chr.	py 33	IPO-0	90128	B30P84	VK98014	IPO428-2	Dintel	Katsiaar	P95873	EC1	89148-09	99183	99189[b]	IPO-C	USA618	89177
BLB	Blb89-256-3	*Rpi-blb3*	4	R	R	R	R	R	R	R	R	R	R	R	R	S	S	S	S
BLB	207TG11-1	*Rpi-abpt*	4	R	R	R	R	R	R	R	R	R	R	R	R	R	S	S	S
BLB	AM3778-16	*R2-like*	4	R	R	R	R	R	R	R	R	R	R	R	R	S	S	S	S
DMS	Cebeco44158-4	*R2*	4	R	R	R	R	Rq	R	Rq	R	R	R	R	R	S	S	S	S
	cv. Desiree	...	...	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
	cv. Bintje	...	...	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S

[a] Differential specificity is in bold. Chr. = chromosome, S = susceptible, R = resistant, and Rq = quantitatively resistant.

[b] Isolate differentially recognized.

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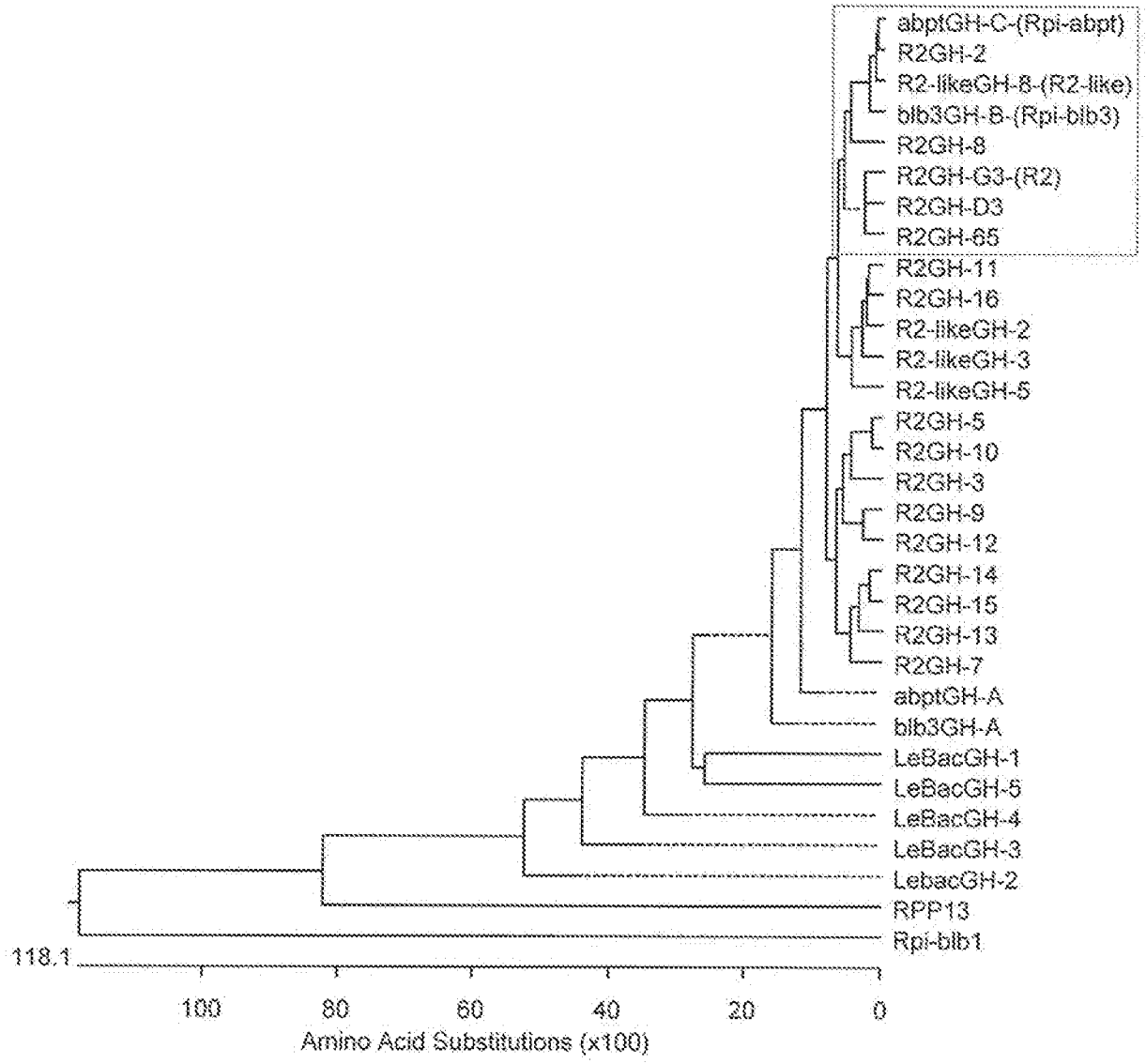


Fig. 4. Phylogenetic tree at the amino acid level of Rpi-blb3; resistance (*R*) gene homologues (RGH) amplified from late-blight-resistant potato clones harboring *Rpi-abpt*, *R2-like*, or *R2*; RGH present on the tomato Bac clone AF411807, *RPP13-Nd*, and *Rpi-blb1*. The group containing Rpi-blb3, Rpi-abpt, R2, and R2-like is boxed.

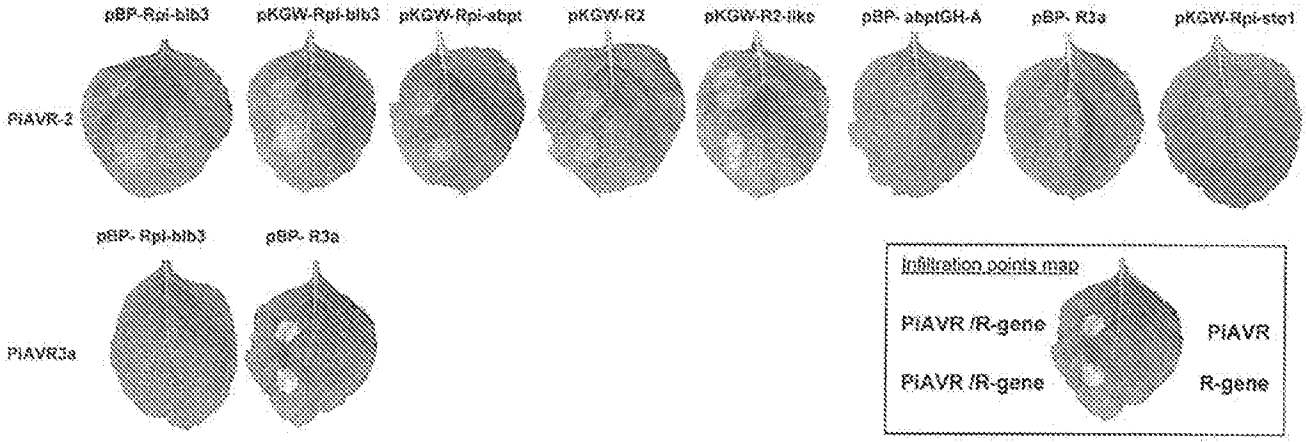


Fig. 5. *Agrobacterium*-mediated coexpression of *PiAvr2* or *PiAvr3a* together with *Rpi-blb3*, *Rpi-abpt* , *R2*, *R2-like*, *Rpi-sto1*, the nonfunctional *abptGH-A*, or *R3a*. Different infiltration points are indicated on the map. On each leaf, four infiltration spots were performed, duplicated co-infiltration of the avirulence (*Avr*) and resistance (*R*) gene on the left, *Avr* on the top right and *R* gene on the bottom right. *Rpi-blb3*, *Rpi-abpt*, *R2*, *and R2-like* in combination with *PiAvr2* as well as *R3a* and *PiAvr3a* induced the hypersensitive response. Pictures were taken 6 days postinfiltration.

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zipper (LZ)-NBS-LRR R proteins (Fig. 6). Interestingly, with respect to known functional R proteins, Rpi-blb3, Rpi-abpt, R2, and R2-like share the highest similarity (34.9% amino acids) with RPP13 from *Arabidopsis thaliana* (Bittner-Eddy et al. 2000), which confers resistance to *H. arabidopsidis* isolates containing *ATR13*. The highest similarity with RPP13 resides in the NBS domain, with 49.3% sequence identity, and the lowest within the LRR domain (34.3%). The LRR domains of Rpi-blb3, Rpi-abpt, R2, and R2-like are highly homologous and comprise 14 imperfect repeats (Fig. 6). The LZ and NBS domains are more polymorphic, those of R2 being the most divergent. Rpi-abpt and R2-like are identical except for the sequence between LRR2 and LRR3, where Rpi-abpt contains a stretch of amino acids that is identical to that of R2. The LRR domain of R2 is identical to that of Rpi-abpt (Fig. 6).

Alignment of the nucleotide sequences of the four functional genes and those of four additional *Blb3GHs* and subsequent analysis of informative polymorphic sites (IPS), (i.e., sites where two or more genes carry the same nucleotide) (Parniske et al. 1997) reveals clear blocks of sequence affiliation between different members of the gene family (Supplementary Fig. S2), indicating that sequence exchange events between *Blb3GHs* have been involved in the evolution of the gene family. Interestingly, the observed sequence affiliations in the 5′ terminal half of the genes extend throughout the LZ and NBS domains whereas sequence affiliations in the LRR domain suggest exchange of specific combinations of LRR, underlining the modular nature of R proteins.

In an attempt to assess the biological relevance of the observed sequence exchange events in relation to resistance spectrum, detached leaves of the parental clones harboring *Rpi-blb3* (Blb99-256-3), *Rpi-abpt* (707TG11), *R2* (Cebeco44158-4(MaR2)), and *R2-like* (AM3778-16) were inoculated with 16 different *P. infestans* isolates (Table 2). For 15 isolates, all four clones displayed the same specificity. However, one isolate, PIC99189, displayed a differential interaction; Blb99-256-3, AM3778-16, and Cebeco44158-4 (MaR2) were susceptible to this isolate whereas 707TG11-1 was resistant (Table 2). Previously, it was reported that 707TG11-1 contained tuber-specific *R1-like* (Park et al. 2005d) and foliage-specific *R3-haplotype* (Huang 2005). To dissect the resistance specificity of 707TG11-1, we inoculated 48 F1 progeny plants of the *Rpi-abpt* mapping population RH4x103 with isolates 90128 and PIC99189. For both isolates, a clear 1:1 segregation of resistance was observed but there was no correlation between resistance to both isolates, confirming that the resistance to isolate PIC99189 is, indeed, the result of a second gene other than *Rpi-abpt*, and not located on LG IV.

In order to confirm the functionality of *Rpi-abpt*, *R2-like*, and *R2*, transgenic Desiree were generated. Detached leaves of six, five, and two independent transgenic lines expressing *R2-like*, *Rpi-abpt*, and *R2*, respectively, were inoculated with *P. infestans* isolates IPO-0 and IPO-C. All transformants reacted with an HR to isolate IPO-0 and were susceptible to isolate IPO-C (Supplementary Fig. S3). This result confirms that *Rpi-abpt*, *R2-like*, and *R2* are functional *R* genes with expected specificity to *P. infestans* isolates. Moreover, the use of the *N. benthamiana* transient complementation assay as an efficient tool for rapid functional screen of putative *R* genes is verified.

DISCUSSION

In this study, we describe the cloning and functional characterization of four homologous *R* genes from the major late blight resistance locus on LG IV of potato. Previously, marker order and allelic conservation suggested that *Rpi-blb3*, *Rpi-abpt*, *R2*, and *R2-like* reside at the same locus on LG IV (Park et al. 2005a, b, and c). *Rpi-blb3* and *Rpi-abpt* were cloned using a map-based cloning approach whereby physical maps of both loci were generated by screening appropriate BAC libraries with a highly linked molecular marker, followed by subsequent rescreening of the libraries with novel BAC end markers until the outer ends of the BAC contigs exceeded the genetic boundaries of the *R* loci. In the case of *Rpi-blb3*, two candidate NBS-LRR genes were identified and subcloned into a binary vector and targeted for complementation analysis through stable transformation of a susceptible potato cultivar.

For *Rpi-abpt*, an NBS-LRR candidate was cloned from the BAC using a homology-based PCR cloning strategy using primers designed on the functional *Rpi-blb3* gene. In addition, complementation was carried out using an *Agrobacterium*-mediated transient expression assay in *N. benthamiana* followed by detached leaf assays with appropriate *P. infestans* isolates (H. Rietman, *unpublished data*).

Development of the transient complementation assay together with the successful in planta expression of *Rpi-abpt* and other *R* genes (H. Rietman, *unpublished data*) using the DNA regulatory elements of *Rpi-blb3* facilitated the rapid cloning of *R2* and *R2-like* through an efficient functional allele-mining strategy. A prerequisite for such an allele-mining strategy is prior knowledge of candidate gene sequences at the locus of interest. Observations within the family *Solanaceae* indicate that *R* gene clusters are conserved across species, thus enabling comparative genomics (Grube et al. 2000; Huang, 2005; van der Vossen et al. 2005). As more and more *R* genes are mapped and cloned, the greater is the chance that novel *R* genes reside at known and well-characterized loci. Moreover, ongoing potato and tomato genome-sequencing projects by international consortia promise to provide a complete survey of the distribution of *R* gene clusters in the family *Solanaceae*, enabling even faster cloning of *R* genes through allele-mining strategies.

Interestingly, with respect to known functional R proteins, Rpi-blb3, Rpi-abpt, R2, and R2-like share the highest similarity (34.9% amino acids) with RPP13 from *A. thaliana* (Bittner-Eddy et al. 2000), which confers resistance to *H. arabidopsidis*. Mining of *RPP13* alleles in a diverse set of *A. thaliana* accessions revealed both extreme sequence diversity and functional diversity in pathogen recognition (Rose et al. 2004). In line with this observation, a significant excess of amino acid polymorphism was localized within the LRR domain of *RPP13*. In the case of *Rpi-blb3*, *Rpi-abpt*, *R2*, and *R2-like*, the LRR domains are nearly identical despite significant polymorphism at the coiled-coil and NBS domains, suggesting conservation of function; and, indeed, we have shown that all four proteins interact with the same effector protein, PiAVR2, which was recently cloned through a map-based cloning approach (E. Gilroy, *personal communication*). Although the origin of *Rpi-abpt* and *R2-like* remain unclear, this finding is a second example of how *R* gene recognition specificities can be conserved across *Solanum* spp. originated from different potato superseries; in this case, *S. bulbocastanum* (*Rpi-blb3*) belongs to the *Potato Stellata Bulbocastana* and *S. demissium* (*R2*) to the *Potato Rotata demissa*. Recently, we demonstrated both gene conservation and specificity conservation between the *S. bulbocastanum*-derived gene *Rpi-blb1* and the *S. stoloniferum*- and *S. papita*-derived orthologues *Rpi-sto1* and *Rpi-pta1*, respectively (Vleeshouwers et al. 2008). Despite apparent positive selection in the LRR domain, the LZ and NBS domains are more polymorphic, those of R2 being the most divergent. Clear blocks of sequence affiliation between the four functional R proteins and additional *Rpi-blb3* gene homologs suggest exchange of LZ, NBS, and LRR domains and also of specific LRR, underlining the modular nature of these proteins. *R2* has a particular gene structure likely to be the

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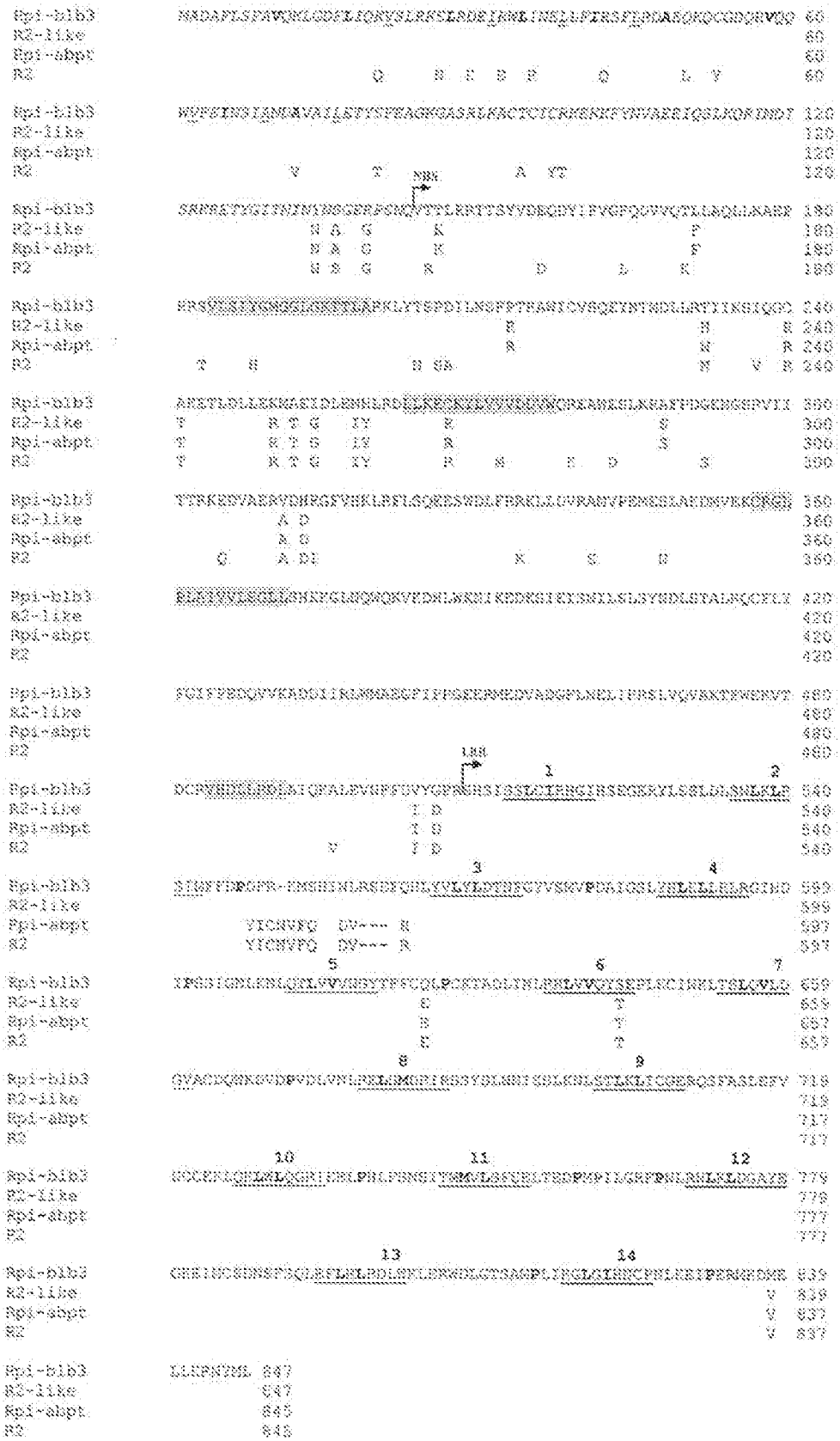


Fig. 6. Alignment of the deduced protein product encoded by the functional genes *Rpi-blb3*, *Rpi-abpt*, *R2-like*, and *R2*. The full amino acid sequence of *Rpi-blb3* and in comparison, differing amino acids of *Rpi-abpt*, *R2-like*, and *R2* are shown. In italics is the leucine zipper domain with the first and the fifth hydrophobic residues from the heptads in bold and underlined, respectively. Positions of the nucleotide binding (NB)-ARC and leucine-rich repeat are indicated by arrows. The NB-ARC domain has its characteristic conserved domains highlighted in gray (kinase 1a, kinase 2, and kinase 3). The leucine-rich repeat domain is composed of 14 repeats with the motif xxLxLxxx.

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product of a relatively recent recombination event. The LZ-NBS domain (nucleotides 1 to 1,149) is highly affiliated to that of the nonfunctional *R2-GH-D3*, whereas the LRR domain (nucleotides 1,059 to 2,544) is highly affiliated to that of *Rpi-abpt* and the nonfunctional *R2-GH-65*, revealing a sequence exchange track between nucleotides 1,059 and 1,149. Sequence exchange between RGH has been proposed as a major mechanism shaping *R* gene diversity in plants (Kuang et al. 2004; Meyers et al. 1999; Michelmore and Meyers 1998). Interestingly, the approximate position of this recombination site corresponds to the one described by Rose and associates (2004) for the *RPP13* allele *ksk-2*, a region between the conserved GLPL and VHD motifs of the NB-ARC domain. This is in line with the expectation that, in multidomain proteins, sequence exchange tracks will be concentrated at specific sites that separate domains with independent functions.

Preliminary results from ongoing *Rpi-blb3* allele-mining studies in *Solanum* germplasm indicate that *Rpi-blb3* is present in several other species (e.g., *S. brachistotrichum* and *S. pinnatisectum*). In addition several *Rpi* genes recently identified in our laboratory (data not shown) also map to the *Rpi-blb3* interval. The quantitative trait locus (QTL) for late blight resistance (Pi-QTL) (Sandbrink et al 2000), explaining 40% of the phenotypic variation, was mapped to the *Rpi-blb3* locus and renamed *Rpi-mcd1* (Tan et al. 2008). Similarly, high-resolution mapping data of the major Pi-QTL identified in cv. Stirling strongly suggest that this Pi-QTL is also an *Rpi-blb3* homologue (Hein et al. 2007) (I. Hein, *personal communication*). In addition, various other examples of Pi-QTL on the short arm of LG IV have been described (Bradshaw et al. 2004, 2006; Leonards-Schippers et al. 1994; Sandbrink et al. 2000).

Rpi-blb3, Rpi-abpt, R2, and R2-like specifically detect PiAVR2. Do the Rpi-blb3 homologues putatively underlying the Pi-QTL described above also recognize PiAVR2 homologues? If so, do they weakly recognize an allele that is conserved throughout *P. infestans* or do they recognize multiple alleles, thus resulting in nonrace-specific resistance?

Current models for NB-LRR R proteins suggest a dual role for the LRR domain, not only as recognition specificity determinant but also as a repressor of inappropriate NB activation (Belkhadir et al. 2004). Furthermore, evolutionary analyses of R proteins and their structure has shown selection pressure on the LRR region but also on other domains within R proteins which, in light of the dual function, may point toward coevolution in R proteins to establish and maintain recognition of effectors while maintaining the inhibitory function (McDowell and Simon 2006). Our findings are slightly different, contradictory, indicating conservation in the LRR domain but more variation in the NBS domain, that may point toward different signaling pathways or additional effectors being recognized by these R proteins.

MATERIALS AND METHODS

Plant material and *P. infestans* isolates.

In this study, we used the four resistant parental clones Blb99-256-3 harboring *Rpi-blb3* (Park et al. 2005a), 707TG11-1 *Rpi-abpt* (Park et al. 2005c), Cebeco44158-4 (MaR2), *R2*, and AM3778-16 *R2-like* (Park et al. 2005b). F1 progeny plants of population RH4x103 were derived from a cross between the late-blight-resistant clone 707TG11-1 and the diploid potato clone RH89-039-16 (Park et al. 2005c). The susceptible potato cv. Desiree was used for *Agrobacterium*-mediated transformation. The characteristics and origin of *P. infestans* isolates used in this study are indicated in Supplementary Table S3.

BAC library construction.

Clones Blb99-256-3 and 707TG11-1 were used as DNA sources for the construction of BAC libraries. High molecular weight DNA preparation and BAC library construction were carried out as described previously (Rouppe van der Voort et al. 1999). Approximately 74,000 clones with an average insert size of 85 kb, corresponding to eight genome equivalents, were obtained per library. The BAC clones were stored as bacterial pools containing approximately 700 to 1,000 white colonies. These were generated by scraping the colonies from the agar plates into Luria-Bertani (LB) medium containing 18% glycerol and chloramphenicol at 12.5 $\mu g\ ml^{-1}$ using a sterile glass spreader. These so-called super pools were stored at −80°C. Marker screening of the BAC libraries was done, first by isolating plasmid DNA from each pool using the standard alkaline lysis protocol, and PCR was carried out to identify positive pools. Bacteria corresponding to positive pools were diluted and plated on LB agar plate containing chloramphenicol (12.5 $\mu g\ ml^{-1}$). Individual white colonies were picked into 384-well microtiter plates and single positive BAC clones were subsequently identified by marker screening as described by Rouppe van der Voort and associates (1999). Names of BAC clones isolated from the super pools carry the prefix Blb (e.g., Blb25) or TG (e.g., TG9).

Subcloning of candidate genes.

Candidate RGH were subcloned from BAC clone BLB25 and TG9 as follows. Aliquots of approximately 1 µg of BAC DNA were digested with 0.03 U of *Sau*3AI restriction enzyme for 10 min. The partially digested BAC DNA was subjected to electrophoresis at room temperature in 0.5× Tris-acetate EDTA using a linear increasing pulse time of 1 to 10 s and a field strength of 90 V cm^{-1} for 6 h. After electrophoresis, the agarose gel was stained with ethidium bromide to locate the region of the gel containing DNA fragments of approximately 10 kbp in size. This region was excised from the gel and treated with GELASE (Epicentre Technologies, Madison, WI, U.S.A.) according to the manufacturer's instructions. The size-selected DNA was ligated to the *Bam*HI-digested and dephosphorylated binary vector pBINPLUS (van Engelen et al. 1995) followed by transformation to ElectroMAX *Escherichia coli* DH10B competent cells (Life Technologies, Paisley, U.K.).

DNA sequencing and computer analysis.

BAC clone sequencing was carried out using a shotgun cloning strategy. Sequencing reactions were performed using a dye terminator cycle sequencing reaction kit (Perkin-Elmer, Pi Biosystem, Warrington, U.K.), M13 universal forward and reverse primers, and an ABI377 automated sequencer (Applied Biosystem, La Jolla, CA, U.S.A.). Sequence contigs were assembled using the STADEN sequence and analysis program (Dear and Staden 1991).

The binary constructs were sequenced using a primer walking strategy (700 by 700 bp). Gene structures were predicted using FGENESH++ (Softberry) and protein sequences were deduced by translation of the ORF using the standard genetic code. Multiple sequence alignments were conducted using CLUSTALX 1.81 (Thompson et al. 1997). The search for genes homologous to *Rpi-blb3* was carried out using the Basic Local Alignment Search Tool (BLAST). Conserved domains were identified using Swiss-Prot (InterProScan, EMBL-EBI, ExPASy, SAPS).

Resistance assay.

Detached leaf assays were used to determine the resistance phenotypes of primary transformants of potato and transiently

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transformed *N. benthamiana* leaves. For complementation analyses, primary transformants were tested with isolates IPO-0 and 90128 (Fig. 2). Inoculum preparations and inoculations were performed as described by Vleeshouwers and associates (1999). Six days after inoculation, plant phenotypes were determined. Leaves showing no symptoms or a localized necrosis at the point of inoculation were scored as resistant and those with clear sporulating lesions (visible by naked eye) as susceptible.

Transient complementation in *N. benthamiana*.

Infiltrations of *Agrobacterium* strains carrying the candidate EGH were performed in *N. benthamiana*. Recombinant COR308 *Agrobacterium tumefaciens* cultures were grown in LB medium (10 g of bacteriological peptone, 10 g of NaCl, and 5 g of yeast extract in 1 liter of milli-Q water) supplemented with tetracycline at 5 mg/liter and kanamycin at 50 mg/liter. After 1 or 2 days, a calculated amount of culture (according to an optical density at 600 nm [OD_{600}] of 0.5) was transferred to YEB medium (5 g of beef extract, 5 g of bacteriological peptone, 5 g of sucrose, 1 g of yeast extract, and 2 ml of 1 M $MgSO_4$ in 1 liter of milli-Q water) supplemented with kanamycin for all strains. After 1 day, overnight cells were centrifuged at 3,500 rpm and resuspended in MMA medium (20 g of sucrose, 5 g of MS salts, and 1.95 g of morpholineethanesulfonic acid) supplemented with 1 ml of 200 mM acetosyringone to a final OD_{600} of 0.2 and infiltrated into leaves of 4-week-old plants with a 3-ml syringe. After 2 days, infiltrated leaves were detached and inoculated with *P. infestans* strains IPO-C and PY23 in detached-leaf assays. HR or *P. infestans* sporulation was observed between 4 and 8 days postinoculation.

***Agrobacterium*-mediated coexpression in *N. benthamiana*.**

pBP-Rpiblb3, *pKGW-Rpi-blb3*, *pKGW-Rpi-abpt*, *pKGW-R2*, *pKGW-R2-like*, *pKGW-Rpi-sto1*, *pBP-abptGH-A*, and *pBP-R3a* were transferred to *A. tumefaciens* AGL1 (Lazo et al. 1991) containing the helper plasmid pVirG and pSoup (van der Fits et al. 2000). Recombinant *A. tumefaciens* cultures were grown in LB medium supplemented with tetracycline at 5 mg/liter, carbenicillin at 100 mg/liter, chloramphenicol at 75 mg/liter, and kanamycin at 50 mg/liter. The avirulence effectors were cloned into the pGRAB vector (kindly supplied by Sean Chapman, SCRI, Scotland, U.K.) and transferred to the same *A. tumefaciens* AGL1. Recombinant *A. tumefaciens* cultures were grown in LB medium supplemented with chloramphenicol at 30 mg/liter and kanamycin at 50 mg/liter. The same protocol as for the transient complementation was used for the subcultures. *PiAVR Agrobacterium* culture and *Rpi-gene Agrobacterium* culture were mixed at a final OD_{600} of 0.1 for each and infiltrated into leaves from 5- to 7-week-old plants. Specific recognition of the *AVR* gene by the *R* gene induces HR from 5 days postinfiltration.

Transformation of susceptible potato cultivar.

Binary plasmids harboring the candidate genes *Blb3GH-A*, *Blb3GH-B*, *AbptGH-A*, and *AbptGH-B* were transferred to *A. tumefaciens* COR308 (Hamilton et al. 1996) and the constructs pKGW-Rpi-abpt, pKGW-R2-like, and pKGW-R2 in AGL1 (Lazo et al. 1991). After verifying their stability in *Agrobacterium* spp., these clones were transformed to the susceptible potato cv. Desiree. Overnight cultures of the transformed *A. tumefaciens* strain were used to transform internodal cuttings from in vitro grown plants (Heilersig et al. 2006). A total of 100 to 200 explants were used for each transformation. Three to eight plants per transformant were transferred to the greenhouse.

Allele mining.

Primers of 32 nucleotides were designed on the Rpi-blb3 sequence, with the forward primer beginning at the start codon (Blb3-start) and the reverse primer beginning at the stop codon (Blb3-end) (Table 1). The BAC clone TG92 containing *Rpi-abpt* and the genomic DNA of the parental resistant genotype AM-(3778-16) containing *R2-like* were used as template in a long-range PCR reaction (95°C for 2 min 40 s; 30× [94°C for 20 min, 56.8°C for 25 min, and 64.3°C for 7min], and 64.3°C for 25 min) using the high-fidelity DNA polymerase *PfuTurbo* (Stratagene, La Jolla, CA, U.S.A.). PCR products were separated on agarose gel and purified using the QIAquick gel extraction kit from Qiagen (Hilden, Germany). The purified pool of R2-likeGH ORF and the purified Rpi-abpt ORF were used in a BP reaction together with the donor plasmid pDONR 221 according to the protocol described by Untergasser (2006b). Subsequently, the BP reaction products were transferred to DH10B *E. coli* competent cells and plated on LB agar plates containing the antibiotic kanamycin at the concentration of 100 μg/ml. Colonies were cultured in LB liquid medium supplemented with kanamycin. Plasmid DNA was subsequently extracted by mini-prep protocol adapted from Sambrook and associates (1989) using the P1, P2, and P3 solutions from Qiagen. The first and last 700 bp of plasmids containing R2-likeGH ORF were sequenced and classified. Then, one clone of each group was entirely sequenced and compared with the *Rpi-blb3* sequence. The clones sharing high similarity with *Rpi-blb3* were cloned into the destination vector pKGW-MG (kindly provided by Andreas Untergasser) in between *Rpi-blb3* regulatory elements via a multiple LR reaction with the *Rpi-blb3* promotor in pDONR-P4P1R, the *Rpi-blb3* terminator in pDONR-P2RP3, the ORF of interest in pDONR221, and the binary destination using the protocol of Untergasser (2006a). The final expression constructs were then transferred to *A. tumefaciens* COR308 after the configuration of the constructs was confirmed by PCR.

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