potato growers are best served with resistant potato cultivars to these nematode species. The successful introgression of resistance to potato cyst nematodes can be taken as an example for the control of root-knot nematodes.

We have shown in Chapters 5 and 6 that the resistance is controlled by a single locus, effective to M. chitwoodi and M. fallax. This means that the resistance to both species is controlled by the same gene or that the resistance locus contains two closely linked R-genes. Both hypotheses are liable, since R-genes are frequently organised in gene clusters and examples of resistance genes acting to distinct pathogen species are known (Van der Vossen et al. 2000). In both cases the monogenic and dominant inheritance of the resistance provides the potato breeder the opportunity to develop resistant cultivars in a relative short period of time. Compared with M. chitwoodi and M. fallax the resistance to M. hapla is more difficult to incorporate into new cultivars. Although we did not always find more than one resistance gene it is evident that more factors play a role in the resistance to M. hapla.

Durability of resistance

In order to predict the durability of the resistance genes and to develop an appropriate breeding strategy it is important to have knowledge of the virulence present across nematode populations. Resistance breaking pathotypes are often a major concern in breeding programmes. In the resistance breeding against P. infestans this concern led to avoiding the introduction of major R-genes and to select for horizontal resistance only. The durability of resistance is depending on the resistance breaking ability of the pathogen and the presence of virulence in the population. Compared to many aerial pathogens the resistance breaking ability of potato cyst nematodes and root-knot nematodes is lower, because they are soil-borne and sedentary and therefore the rates of gene flow and spread of virulence alleles will be low. The durability of a major resistance gene against the potato cyst nematodes has been shown in the United Kingdom where potato cultivars with the H1 resistance gene from S. tuberosum ssp. andigena have been widely grown over 30 years without selecting virulent populations so far (Turner & Flemming 2000). For the Globodera spp. it has been pointed out that the different pathotypes already existed in the initial nematode population in Europe. The use of resistance genes like the H1-gene in the breeding programme and subsequently in the cultivars, lead to a change of virulences in the population, more than only mutation in a virulent nematode (Folkertsma et al. 2001). Despite the shorter generation time of Meloidogyne spp., it is feasible that the root-knot nematode has a comparably poor resistance breaking ability as the potato cyst nematode. Consequently the durability of the resistance to the root-knot nematodes would also mainly depend on the variability in virulence of the population already present in the soil. In contrast to the potato cyst nematode of which only a small proportion of the nematode genepool has been introduced in Europe, the root-knot nematode populations are presumably not a result of a few small introductions into specific regions. When the virulence factors across nematode populations are known, the breeder is able to use effectively these populations to screen other wild Solanum species in order to detect other resistance genes.

Durability of the qualitative resistance to M. chitwoodi and M. fallax

In Chapters 5 and 6, we showed that occasionally egg-masses of M. chitwoodi and M. fallax were formed on the resistant genotypes of the mapping population. This has also been found in other studies (Janssen 1997a; van der Beek 1997; Austin et al. 1993; Brown et al. 1989,1991,1995). A possible explanation is that these egg-masses are a result of avirulent nematodes that escaped the resistance response in the plant. However, Janssen et al. (1998) showed that the nematode populations are a mixture of virulent and avirulent genotypes. The ease of selecting a virulent population of M. chitwoodi by Janssen et al. (1998) raise questions about the durability of the $R_{Mc1\text{-}6m}$ gene. Although their research was not performed in a field assay, their results may have great implications for the durability of all R_{Mc1} genes mapped in the experiments described in this thesis. The change of the allele frequency in time is influenced by 1) the meiotic parthenogenetic reproduction, 2) the multiplication and 3) the decrease during the growth of other crops. The shift in virulence might also retard when the fecundity of virulent isolates is lower, as has been shown in M. incognita (Castagnone-Sereno et al. 1994). The amount of produced egg masses of laboratory-selected virulent isolates of M. incognita differed between susceptible and Mi-resistant tomato clones, in contrast to virulent field isolates, suggesting a different genetic control of the virulence. We have not found indications that the fecundity of virulent isolates was different on susceptible plants compared with resistant plants (Chapter 6).

The variation in virulence in M. chitwoodi populations could also be demonstrated by the interaction to pepper lines containing Me resistance genes (Berthou et al. 2003). The Co population appeared to be avirulent to these Me resistance genes, similar to its interaction to the genes $R_{Mc1\text{-}6m}$ (Chapter 5), $R_{Mc1\text{-}9g8}$ and $R_{Mc1\text{-}8s}$ (Chapter 6). Two other Dutch M. chitwoodi populations showed virulence to the Me resistance genes and remarkably the Me resistant pepper was susceptible to the tested M. fallax population (Berthou et al. 2003). Our results did not show a differential interaction of M. chitwoodi and M. fallax to the three mapped R_{Mc1} genes (Chapters 5 & 6). The results of Berthou et al. (2003) and Chapter 6 show that more virulence factors may already be present in the Dutch M. chitwoodi populations. The amount of virulence factors present is however still unknown. Resistant cultivars can be used to select virulent mono-female lines. The different mono-female lines can subsequently be used to categorise the different virulence factors and give the breeder a tool for the selection of new sources of resistance in the wild Solanum species.

Durability of the quantitative resistance to M. hapla

The resistance to M. hapla described in Chapters 2 and 4 shows quantitative variation. For a long time quantitative resistance was considered to be race-non-specific, durable and equated with polygenic resistance. However in barley, cultivar by isolate interactions have been identified (Qi, 1998), and a 'minor gene-for-minor gene' model was postulated. In soybean three out of four QTLs detected for resistance to the soybean cyst nematode were race-specific (Concibido et al. 1997). These results indicate that it is likely that minor gene-for-minor gene interactions occur in quantitative resistance. In Chapter 3 the interaction of the two QTLs of S. chacoense $R_{Mh\text{-}chc}A$ and $R_{Mh\text{-}chc}B$ and eight M. hapla populations is described. The race B populations of M. hapla showed to be virulent to both resistance genes. Also Janssen et al.

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(1997a) demonstrated the presence of virulent populations of M. hapla towards different Solanum species. In their research the tested M. hapla were not classified as cytological races A or B. Within race A only one population showed interaction with the $R_{Mh\text{-}chc}B$ gene. This provides a slight indication of the existence of a 'minor gene-for-minor gene' model, although for a concluding proof more research is required. The question of whether the race-non-specific QTL may also be race-specific can only be answered when a larger number of races are tested. If the 'minor gene-for-minor gene' model also refers to the resistance to M. hapla, the durability of this resistance might be comparable to the durability of the qualitative resistance to M. chitwoodi and M. fallax.

Pathotype scheme

The durability of resistance can be extended by the application of different R-gene combinations. Bioassays with a range of different resistant genotypes on the unselected nematode population are required to gain insight in the variation of virulence already present. In this thesis the existence of virulence within the M. hapla and M. chitwoodi populations is demonstrated with a limited amount of resistant Solanum genotypes. Van der Beek (1997) and the results of Chapter 3 show that the populations of M. hapla are genetically heterogenic. In addition, the variation within the populations of M. hapla makes the genetic analysis of the resistance more difficult.

A pathotype scheme with differential clones, comparable to the international pathotype scheme of the potato cyst nematodes (Kort et al. 1977) can help to find new resistance genes in wild species. Such a pathotype scheme describes nematode populations that can or cannot reproduce on a given host genotype. Until now the lack of resistances within the Solanum species did not allow classification of pathotypes. The differences between populations that represent specific pathotypes can be very small and difficult to quantify in a bioassay. Therefore, a pathotype scheme should not only be based on arbitrary use of Pf/Pi values. The classification of the pathotypes Pa2 and Pa3 of G. pallida is based on the ability to reproduce on the susceptible S. vernei hybrid 62.33.3 as indicated by Pf/Pi. This appeared to be independent from the genetic characterization of the G. pallida populations (Folkertsma et al. 1996a). In contrast to the G. rostochiensis populations with a restricted genetic variation, the G. pallida populations are more variable and comprise mixtures of virulence gene frequencies. Furthermore the differential clone S. vernei hybrid 62.33.3 is not fully resistant to the Pa2 pathotype used in the official trial (pers. com. A.N.O. Rookmaker, Averis Seeds). Therefore, the pathotype scheme of the potato cyst nematodes should be reconsidered. The gene pool similarity concept as proposed by Bakker et al. (1993) would be an appropriate tool to distinguish the different nematode populations. The gene pool similarity concept implies that in absence of selection pressure the molecular similarity of the populations will reflect on the virulence loci. This concept was mainly illustrated for the control of potato cyst nematodes, having a poor reproduction capacity and poor dispersal ability. Compared to the Globodera spp. the Meloidogyne spp. will have a higher reproduction capacity due to more generations during a growing season and the broader host range. Nevertheless this concept can also apply to the Meloidogyne spp.. Therefore, molecular markers are needed to state that one pathotype share the same alleles for reproduction on a given plant genotype. Breeders can subsequently use

distinct genetic clusters of the nematode populations as a guide to screen for broad-spectrum resistance. The classification of the two races of M. chitwoodi has also been based on solely the Pf/Pi value (Santo & Pinkerton 1985). The results of Chapter 6 show a similar response of the race 2 populations and the virulent race 1 population. Molecular analysis of the M. chitwoodi populations might result in a new classification of the races and pathotypes. A proper characterization of the resistance genes and nematode populations will eventually lead to a better knowledge of the amount of virulence factors present already in the nematode populations.

Recent observations in Malta indicate that the root-knot nematode species M. javanica is an emerging pest for potato cultivation, causing severe galling on the tuber surface (Vovlas et al. 2005). Also the recently described new root-knot nematode species M. minor, causes in potato similar symptoms as M. chitwoodi and M. fallax (Karssen et al. 2004). Future research is required to examine the value of the current set of resistance genes against a wider range of Meloidogyne species.

Development of molecular markers

One of the well known benefits of MAS is an early selection in the breeding programme and avoiding expensive and time consuming bioassays like the bioassay for resistance to root-knot nematodes. Furthermore the molecular marker techniques are not influenced by the environment in contrary to bioassays. Because there is no root-knot nematode resistance available in potato genotypes with an acceptable level of agronomical value, the long way of introgression breeding has to be followed. The use of molecular markers offers the breeder a tool of a very early selection without efforts to generate additional material for a bioassay. The profits of MAS are among others depending on the heritability of the trait and its mode of inheritance. We showed in chapter 5 and 6 that the resistance to M. chitwoodi and M. fallax from the wild species S. fendleri, S. hougasii and S. bulbocastanum have a monogenic inheritance. In practical breeding these simple qualitative phenotypes with high heritability benefit only in the sense of assay efficiency. Furthermore the two mentioned nematode species are quarantine organisms, requiring the bioassay only to be performed under strict regulations. Therefore, many of these bioassays could efficiently be replaced by molecular marker techniques. The resistance to M. hapla from the wild species S. chacoense and S. tarijense have a low heritability. The large environmental variation in the bioassay will lead to frequent loss of resistance when MAS is not applied as has been shown in Chapter 2. For the development of potato cultivars with improved resistance to M. hapla the potato breeder will benefit much more from molecular markers as a selection tool. Besides these trait by trait benefits, MAS will give the breeder a tool to create combinations of phenotypically similar resistance genes in a variety, so-called pyramiding of resistance genes, as will be discussed later.

Molecular analyses of quantitative resistance

Initial results of bioassays with clones derived from the M. hapla resistant wild species S. chacoense (Chapter 2) and S. tarijense (Chapter 4), demonstrated continuous distribution of resistance scores and a low heritability, suggesting the involvement of QTLs. In contrast to the

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study of monogenic characteristics, a genome wide marker coverage is essential to allow QTL studies. Because both wild species are diploid and crossable with diploid S. tuberosum, the use of a diploid mapping population was obvious. Especially the construction of a genetic map can be performed with much more ease in a diploid population. Compared with a tetraploid population the construction of a genetic map of a diploid population will take less effort. Bradshaw et al. (2004) failed to produce a complete linkage map of a tetraploid mapping population using 38 AFLP primer combinations and 23 SSRs. We produced genetic maps of the S. chacoense and S. tarijense mapping population using ten AFLP primer combinations. The use of a diploid mapping population is also a major advantage for the integration of breeding and research. In this way the ease of mapping and the more rapid introduction of the genes of interest into cultivated potato in diploid way, are combined. In many QTL studies the association found in the tested mapping population between the markers and a QTL or even a major gene can not always directly be extended to other populations with a different genetic background. This drawback is not expected in the followed approach of a combined genetic QTL study and the introgression of the resistance.

Molecular analyses of qualitative resistance

In order to map the genes involved in the monogenic inherited resistance the construction of a complete genetic map is not necessary. Because the resistance against M. chitwoodi and M. fallax from the wild species S. fendleri (Chapter 5), S. hougasii and S. bulbocastanum (Chapter 6) was inherited monogenically, only a partial linkage map of one of the four homologous chromosomes in the vicinity of the resistance gene needed to be constructed. Therefore, the problematic construction of a complete genetic map of a polyploid mapping population could be avoided. With the BSA approach (Michelmore et al. 1991) a large amount of AFLP primer combinations could be tested in a short period of time, resulting in a linkage group of 11 AFLP markers in the vicinity of the $R_{Mc1\text{-}fen}$ locus (Chapter 5) and a linkage group of 14 AFLP markers in the vicinity of the $R_{Mc1\text{-}hou}$ locus (Chapter 6). A disadvantage of constructing only a partial linkage map is the anticipated lack of markers that is homologous (co-migrating to AFLPs with known chromosomal positions) necessary to align the linkage group to linkage groups in reference maps. In Chapter 5 the method of map alignment using co-migrating AFLP markers as suggested by Rouppe van der Voort et al. (1997a) indeed failed to align the linkage group. By sequencing the linked AFLP markers and application of the sequence information for the design of primers, we were able to assign the resistance gene to the chromosome number.

Implementation of molecular markers in a potato breeding programme

The implementation of molecular markers in a breeding programme of potato depends on many factors. The costs per data point, the breeding goal and the clonal generation to apply selection criteria play an important role in the decision to utilise molecular markers in a breeding programme. Although most recent molecular studies are performed with molecular markers like AFLP and SSRs, these techniques are not suitable for MAS in a commercial breeding programme. The AFLP markers used in our genetic studies are very efficient in genetic studies but technically demanding and expensive. Therefore, AFLP markers should be converted into a single locus PCR marker, preferably without the necessity of an enzymatic restriction step like

for CAPS markers. MAS should be applicable in both diploid as well as in tetraploid breeding programmes. In order to follow the marker in tetraploid clones, the molecular marker should not give to many bands, preferable only one allele specific amplification product. The markers have to be robust in PCR conditions and low in costs. Recombination between a marker and the gene of interest would nullify the value of the marker. Therefore, markers should be close to the target locus, and all offspring without the marker should be discarded or two markers, on both sides of the target locus, should be used. In an ideal situation the marker is based on the sequence of the gene of interest.
In most potato breeding programmes the selection in the first two years is performed on visually observable characters, such as length of stolons, malformations of the tuber, amount of tubers and tuber size. After one year of field selection, the number of clones is reduced with approximately 90%. Performing MAS after the first field selection will, therefore, drastically reduce the marker genotyping costs. The costs per data point for MAS, however, depend on the volume. Application of MAS either before the development of greenhouse tubers or after the first field selection should be based on the costs per data point versus the costs made for planting, nursing and storage of clones. Besides this high throughput MAS with emphasis on low costs another strategy of implementation of MAS can be perceive: the targeted application of MAS for the development of progenitor lines. To increase the change for offspring with the genes of interest in the latter strategy, the tetraploid progenitor clones should have these genes in at least a duplex way. Because of the relative ease of genetics at the diploid level and the transfer of the majority of the alleles of the diploid parent in the progeny, it is preferred to apply this strategy in diploid crossing parents.

Pyramiding of resistance genes

The application of molecular markers also allows novel and additional goals to be achieved: (1) phenotyping can be replaced by genotyping, and (2) breeding by design (Peleman & Rouppe van der Voort 2003) to generate intended genetic combinations, including the pyramiding of resistance genes (Zhao et al. 2005). When phenotyping is replaced by genotyping, combinations of genes can be distinguished from single genes, although the disease or quality assay result in similar phenotypes. As an example black spot bruising could be mentioned where either lack of thyrosine substrate as well as lack of PPO enzyme activity would equally result in bruising resistance. In this Thesis, a number of resistance genes have been identified, that could be used to design a breeding strategy for more durable resistance. This is also referred to as pyramiding of resistance genes. The hypothesis is that a set of resistance genes in one plant would be harder to overcome by the pathogen, or might have resistance to a broader set of virulence genes in the pathogen. To have a more durable resistance due to combinations of resistance genes, these resistance genes should interact in different ways with the pathogen. For example, a resistance gene preventing the pathogen to enter the plant combined with a resistance gene preventing the formation of spores. An example of broader resistance due to pyramiding is the combination of resistance genes acting against different virulence genes of the potato cyst nematode. The present way to determine the resistance against the potato cyst nematodes is performing a bioassay with a population with unknown virulence genes. In that situation the Gpa2 gene (Rouppe van der Voort et al. 1997b) in a

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potato clone will prevent the avirulent nematodes in the population to multiply, but because of the low frequency of the avirulent alleles in the tested nematode population this potato clone will be scored as susceptible. Together with other genes the Gpa2 gene might be the cause of the difference between the Dutch qualification of 'high resistant' and 'resistant'. Pyramiding of genes can be performed with qualitative genes and QTLs. The implementation of QTLs in a breeding program is depending on the amount of explained variance. QTLs with low explained variances, like $R_{Mh\text{-}chcB}$ (Chapter 2), will not easily be used in a high throughput MAS. Also because $R_{Mh\text{-}chcB}$ did not have an effect on all other M. hapla populations tested (Chapter 3), suggesting a low frequency of the avirulent alleles of these populations. A breeder would prefer to combine QTLs with a higher explained variance, for instance by pyramiding the genes $R_{Mh\text{-}chcA}$ (Chapter 2) and $R_{Mh\text{-}tar}$ (Chapter 4). The resistance genes $R_{Mh\text{-}chcA}$ and $R_{Mh\text{-}tar}$ alone show an insufficient level of resistance. A combination of these two genes might have a broader resistance. A prerequisite is that the two mentioned resistance genes are acting against different virulence genes, which has to be confirmed by future research.

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Summary & Samenvatting

Summary

The development of potato varieties with resistance towards the potato cyst nematode, allowed a dramatic decrease of the use of nematicides. Subsequently the population of the free living nematodes and the root-knot nematodes (Meloidogyne spp.) has increased. Among the root-knot nematodes, three Meloidogyne species are important in the potato cultivation in The Netherlands: M. hapla, M. chitwoodi and M. fallax. The latter two species are the most harmful because they cause malformation in the tubers and are quarantine organisms. Until now no resistant potato cultivars are available. Several wild species of potato show resistance, but several back crossings are required to develop a root-knot nematode resistant potato cultivar. Selection based on molecular markers will speed up this development and will replace the expensive and time consuming bioassays, which have to be carried out under strict quarantine conditions. In this thesis diploid as well as polyploid wild Solanum species are used for the introgression of resistance genes in cultivated potato, and to analyse the inheritance of the various resistances.

Chapter 2 describes the genetic analysis of the diploid wild species Solanum chacoense with resistance against M. hapla and M. fallax. An AFLP based linkage map was constructed, resulting in twelve linkage groups of either parent of the mapping population with a total length of 685 and 596 cM each. Due to the large differences in AFLP patterns between S. chacoense and cultivated potato, chromosome numbers could not be assigned to the linkage groups containing the QTLs. Two QTLs for resistance against the M. hapla population Hi were identified in this population. These two QTLs, $R_{Mh\text{-}chc}A$ and $R_{Mh\text{-}chc}B$, explained 38% and 13% of the total phenotypic variance of the resistance to M. hapla. As a consequence of the large variation in the results of the bioassay, phenotypic selection may lead to the loss of these resistance QTLs. This loss can be prevented by marker assisted selection with the markers flanking the QTLs. The genetic analysis of the resistance against M. fallax resulted in the detection of one QTL explaining 14% of the phenotypic variance. The high level of resistance to M. fallax as observed in the resistant parent was not observed in the progeny. In view of the modest results that can be expected from S. chacoense, we cannot recommend breeding efforts with this germplasm. Better results in genetic analyses and introgression of resistance against M. fallax were achieved in the mapping studies with the wild Solanum species S. fendleri (Chapter 5), S. hougasii and S. bulbocastanum (Chapter 6).

The differential interaction of eight M. hapla populations to the resistance QTLs, $R_{Mh\text{-}chc}A$ and $R_{Mh\text{-}chc}B$ was studied in Chapter 3. The $R_{Mh\text{-}chc}A$ allele allows recognition on virulence factors present in all six tested cytological race A populations. Although the allele $R_{Mh\text{-}chc}B$ also showed a reduction in the amount of formed egg masses of population Ham, this allele had only an additive effect with population Hi, the population initially used for the QTL analysis (Chapter 2). No effect of both alleles was noticed for the two race B populations. Besides the distinctive difference in reproduction between race A and B, both races can now also be divided based on their differences in virulence to the $R_{Mh\text{-}chc}A$ allele. Furthermore, the molecular fingerprints allow too clearly distinguish between the races A and B. Because the resistant parent of the mapping population is susceptible to the race B populations, it is not worthwhile to check the used mapping population for other resistance QTLs. The breeder should therefore search for new sources of resistance to the race B populations in the wild species. Looking at the higher level

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of reproduction, the population Han would be the most appropriate population to be used in that survey.

In Chapter 4 the diploid wild species S. tarijense was used as the source of resistance to M. hapla. The non absolute level of resistance and the continuous distribution of produced egg masses in the segregating offspring, suggests that the resistance is under polygenic control. Therefore a linkage map of the BC1 mapping population was developed as prerequisite of QTL analysis. The assignment of a chromosome number to the linkage groups was simple, because a detailed genetic map was already constructed of the susceptible parent. The linkage groups of the resistant female parent were subsequently aligned to the genetic map of the susceptible male parent using the AFLP markers that were heterozygous in both parents (so-called ab×ab markers). The QTL mapping approach resulted in only one locus, $R_{Mh\text{-}tar}$, on chromosome 7. In contrast to QTL analysis that leads to a broad QTL interval, qualification of the resistance will lead to a more precise map position of the resistance locus. For that case the progeny of the mapping population was arbitrary classified in two groups: 1) a resistant group with the amount of less than1 egg mass and 2) a susceptible group with more than 10 egg masses. Via this approach the $R_{Mh\text{-}tar}$ resistance gene could be located on a distal position of the short arm of chromosome 7, and the low amount of singletons offered an indirect validation of the classification of the resistance phenotypes in discrete groups. The value of the $R_{Mh\text{-}tar}$ gene for the potato breeding is depending on the allele frequency of the associated virulence genes in the M. hapla populations and the geographic distribution of such populations.

In contrast to the research with M. hapla as described in Chapter 2 and 4, the progeny of the mapping populations with resistance against M. chitwoodi and M. fallax (Chapter 5 and 6) could be easily classified as resistant or susceptible. This suggests that resistance is mediated by a single R-gene. The resistance against both nematode species, M. chitwoodi and M. fallax, was absolutely correlated in all three mapping populations, derived from S. fendleri (Chapter 5), S. hougasii and S. bulbocastanum (Chapter 6). In Chapter 5 the monogenic resistance against M. chitwoodi and M. fallax was mapped in an inter-specific and an intra-specific mapping population of the tetraploid wild species S. fendleri. By using the so-called Bulked Segregant Analysis (BSA) method, a large amount of closely linked markers was identified efficiently. In the intra-specific mapping population a remarkably lower amount of linked AFLP markers was found. This can be due to the fact that S. fendleri is a self-fertilising species, showing a lower level of genetic polymorphisms. On the other hand the genetic dissimilarity between the wild species introgression segments and the recurrent potato parents may have caused an elevated level of polymorphic markers in the BC_1 inter-specific mapping population. Besides the larger amount of polymorphic markers, the utilisation of inter-specific mapping populations is an advantage for the breeder, because back-crossings are already made, necessary to introgress the resistance into potato. Based on the sequence of an $R_{Mc1\text{-}fen}$ locus linked AFLP marker, a PCR marker was developed. This marker was used to map its position in a reference map, were it was located on the distal position of the long arm of chromosome 11. The chromosomal position of the $R_{Mc1\text{-}fen}$ locus was confirmed by using the chromosome 11 specific CAPS marker M39b. The $R_{Mc1\text{-}fen}$ gene is located in a cluster of resistance genes, containing the highly related Rmc1 gene and other fungus, virus and nematode resistance genes. This PCR marker,

however, can not yet be used in marker assisted selection because 40% of the progeny with the PCR product will not contain the resistance allele.

Chapter six describes the localisation of resistance against M. chitwoodi and M. fallax in a BC2 population of the hexaploid wild species S. hougasii. Also this study used the BSA method, resulting in a linkage group of 14 AFLP markers and the monogenic resistance locus $R_{Mc1\text{-}hou}$. Similar to the linkage group of S. fendleri, the linkage group of S. hougasii has been positioned on the distal position of the long arm of chromosome 11. The differential interaction between the resistance genes $R_{Mc1\text{-}blb}$, $R_{Mc1\text{-}fen}$ and $R_{Mc1\text{-}hou}$ with six M. chitwoodi populations has been studied. Differences in virulence were not found between race 1 and race 2, but, remarkably, within the two races. Populations Ccl and Cbh seem to have a similar virulence spectrum, while these populations are described as respectively race 1 and race 2. The presence of virulence in the populations will have a negative effect on the durability of the resistance. Because the three resistance genes against M. chitwoodi and M. fallax from the wild species S. bulbocastanum, S. fendleri and S. hougasii are mapped on the same locus and act against the same spectrum of virulence genes, no additional value is expect from the strategy of pyramiding these genes. Consequently, the breeder has to search for new resistance sources against the virulent nematode population Ccl and/or Cbh, in order to develop a potato with broad spectrum resistance.

The resistance genes described in this thesis can be introgressed in potato with the help of molecular markers. Moreover the presence of individual resistance genes can be monitored by molecular markers, which is a prerequisite for pyramiding of R-genes. Pyramiding of the resistance genes may lead to a resistance with a broader spectrum, and/or may offer a more durable resistance, providing that it can be performed with loci with a complementary resistance spectrum.

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Samenvatting

Door de ontwikkeling van aardappelmoeheid resistente aardappelrassen is het gebruik van nematiciden drastisch afgenomen, met als gevolg een opkomst van de vrijlevende nematoden en het worteiknobbelaaltje, Meloidogyne spp.. In Nederland komen drie voor de aardappelteelt belangrijke Meloidogyne soorten voor, M. hapla, M. chitwoodi en M. fallax waarvan de laatste twee het meest schadelijk zijn in de aardappelteelt en bovendien een quarantaine status hebben. Tot nu toe is in de huidige aardappelrassen geen resistentie tegen deze nematoden aanwezig. Verschillende wilde soorten zijn echter wel resistent, maar het vergt nog vele terugkruisingen voordat een worteiknobbelaaltje resistente aardappelras op de markt kan verschijnen. Selectie op basis van moleculaire DNA merkers zal de ontwikkeling van resistente aardappelrassen versnellen en bovendien vervangt deze moleculaire merker analyse de dure resistentietoets die onder quarantaine omstandigheden plaats moet vinden. In deze thesis zijn zowel diploïde als polyploïde wilde soorten gebruikt om resistente aardappelen te ontwikkelen en de resistentie te karteren.

Hoofdstuk 2 beschrijft de genetische analyse van de diploïde wilde soort Solanum chacoense met resistentie tegen M. hapla en M. fallax. Er werden koppelingskaarten met AFLP merkers gemaakt van beide kruisingsouders met als resultaat twee genetische kaarten van 12 koppelingsgroepen met een totale lengte van 685 en 596 cM. Door de grote genetische verschillen tussen deze wilde soort en de cultuuraardappel was het niet mogelijk om op basis van AFLP techniek de chromosoomnummers toe te wijzen aan alle koppelingsgroepen. Er werden twee QTLs gevonden voor resistentie tegen de M. hapla populatie Hi. Deze twee QTL's, $R_{Mh\text{-}chc}A$ en $R_{Mh\text{-}chc}B$, hadden een verklaarde variantie van respectievelijk 38% en 13%. De grote variatie in de resistentietoets is de reden dat fenotypische selectie kan leiden tot verlies van de afzonderlijke resistentiegenen. Dit kan voorkomen worden door de fenotypische selectie te vervangen door merker gestuurde veredeling op basis van de gevonden flankererende merkers. De genetische analyse van de resistentie tegen M. fallax resulteerde in de detectie van één QTL met slechts 14% verklaarde variantie. Bovendien werd de mate van resistentie tegen M. fallax van de resistente ouder niet terug gevonden in de nakomelingen. Betere vererving van de resistentie tegen M. fallax werd verkregen in de mappingpopulaties met resistenties uit S. fendleri (Hoofdstuk 5), S. hougasii en S. bulbocastanum (Hoofdstuk 6).

De differentiele interactie tussen de QTL's $R_{Mh\text{-}chc}A$ en $R_{Mh\text{-}chc}B$ en acht M. hapla populaties werd bestudeerd in Hoofdstuk 3. Het $R_{Mh\text{-}chc}A$ allel herkent de virulentie factoren van alle getoetste cytologische race A populaties. Alhoewel het $R_{Mh\text{-}chc}B$ allel ook een reductie in het aantal gevormde eiproppen laat zien bij de populatie Ham, had dit allel alleen een significant additioneel effect bij populatie Hi, de M. hapla populatie die in Hoofdstuk 2 gebruikt werd voor de QTL analyse. Beide QTL's hadden geen effect op de race B populaties van M. hapla. Naast de het verschil in meiose tussen race A en race B, kunnen de twee race's dus nu ook onderscheiden worden op basis van de virulentie tegen het $R_{Mh\text{-}chc}A$ allel. Ook met moleculaire merkers is een duidelijk onderscheid te maken tussen race A en race B. Omdat de resistente ouder ook vatbaar is voor de race B populaties heeft het geen zin om de mapping populatie te onderzoeken op aanwezigheid van resistentie QTL's. De kweker zal dus op zoek moeten gaan naar nieuwe bronnen van resistentie tegen race B. Gezien de mate van reproductie van

populatie Han, is dit de meest aangewezen populatie om nieuwe bronnen van resistentie op te sporen.

In Hoofdstuk 4 is de diploide wilde soort S. tarijense gebruikt als resistentie bron voor M. hapla. Het niet absolute niveau van resistentie en de continue verdeling van de geproduceerde eiproppen in de nakomelingen, suggereert dat de resistentie in deze wilde soort polygeen is. Daarom werd van de BC1 karterings populatie een volledige genetische kaart, nodig voor QTL analyse, gemaakt. Het toekennen van de chromosoomnummers aan de koppelingsgroepen was in deze populatie eenvoudig omdat van de vatbare ouder al een zeer gedetailleerde genetische kaart bestond. De koppelingsgroepen van de resistente moeder kon vervolgens met ab×ab merkers gekoppeld worden aan de genetische kaart van de vatbare vader. De QTL analyse resulteerde in de detectie van slechts één QTL, $R_{Mh\text{-}tar}$, op chromosoom 7. De resistentie werde ook kwalitatief gekarteerd om zodoende een nauwkeurige locatie van het resistentiegen te krijgne. De nakomelingen van de onderzoekspopulatie werden daartoe in twee groepen verdeeld op basis van een arbitraire indeling van 0-1 eiprop voor de resistente groep en meer dan 10 eiproppen voor de vatbare groep. het $R_{Mh\text{-}tar}$ resistentie gen werd gekarteerd distaal op de korte arm van chromosoom 7. De waarde voor de aardappelveredeling van het $R_{Mh\text{-}tar}$ gen hangt af van de allelfrequentie van de bijbehorende virulentiegen in de M. hapla populaties en de geografische verdeling van deze populaties.

In tegenstelling tot het onderzoek met M. hapla, beschreven in Hoofdstukken 2 en 4, kunnen de nakomelingen van onderzoekspopulaties met resistentie tegen M. chitwoodi en M. fallax, beschreven in de hoofdstukken 5 en 6, eenvoudig ingedeeld worden in een resistente en vatbare groep. De resistentie tegen M. chitwoodi en M. fallax was in alle drie onderzoekspopulaties, S. fendleri (Hoofdstuk 5), S. hougasii en S. bulbocastanum (Hoofdstuk 6), volledig gecorreleerd. In Hoofdstuk 5 is in de tetraploide wilde soort S. fendleri de monogene resistentie tegen M. chitwoodi en M. fallax gekarteerd in een interspecifieke en een intraspecifieke onderzoekspopulatie. Daarbij werd gebruik gemaakt van de Bulked Segregant Analysis (BSA) methode, waardoor zeer efficiënt een groot aantal gekoppelde merkers gevonden werd. In de intraspecifieke karteringspopulatie werd een opvallend lager hoeveelheid gekoppelde AFLP merkers gevonden, hetgeen verklaard kan worden doordat S. fendleri een zelfbevruchtend soort is en daardoor genetisch minder polymorfie bezit. Bovendien kunnen de genetische verschillen tussen de wilde S. fendleri en de cultuuraardappel een oorzaak zijn van de hogere mate van polymorfie in de interspecifieke onderzoekspopulatie. Naast de hogere hoeveelheid merkers is het voor de kweker vooral voordelig om interspecifieke populaties te gebruiken, omdat dan al een begin wordt gemaakt met de vele terugkruisingen die nodig zijn om de resistentie in een kwalitatief hoogwaardig aardappelras te krijgen. Op basis van de sequentie van een aan het $R_{Mc1\text{-}fen}$ locus gekoppelde AFLP merker, werd een PCR merker ontwikkeld. Deze PCR merker werd gebruikt in een referentie populatie waar het gekarteerd werd op de distale positie van de lange arm van chromosoom 11. De locatie van de koppelingsgroep met het $R_{Mc1\text{-}fen}$ gen werd bevestigd door de kartering van het chromosoom 11 specifieke CAPS merker M39b. Het $R_{Mc1\text{-}fen}$ gen ligt in een cluster van resistentiegenen, waaronder de sterk gerelateerde Rmc1 gen en andere schimmel, virus en nematoden resistentiegenen. De PCR merker kan echter nog niet gebruikt worden voor merker gestuurde

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veredeling, omdat in 40% van de nakomelingen met het PCR product het resistentie allel afwezig is.

In Hoofdstuk 6 is de resistentie in de hexaploide wilde soort S. hougasii in een BC2 populatie gekarteerd. Ook hier werd de BSA methode gebruikt en dit resulteerde in een koppelingsgroep met 14 AFLP merkers en de monogene resistentie locus $R_{Mc1\text{-}hou}$. Deze koppelingsgroep werd net als de koppelingsgroep van S. fendleri gekarteerd op de distale positie van de lange arm van chromosoom 11. Vervolgens werd de differentiele interactie tussen de resistentiegenen $R_{Mc1\text{-}blb}$, $R_{Mc1\text{-}fen}$ en $R_{Mc1\text{-}hou}$ met zes M. chitwoodi populaties bestudeerd. Opmerkelijk was dat de virulentie verschillen niet gevonden werd tussen race 1 en race 2, maar binnen de race's. Voorlopig lijken de populaties Col en Cbh een gelijke virulentie spectrum te hebben, terwijl deze populaties beschreven staan als respectievelijk race 1 en race 2. De aanwezigheid van virulentie in de populaties zal de duurzaamheid van de huidige resistentie negatief beïnvloeden. Omdat de drie gekarteerde resistentiegenen tegen M. chitwoodi en M. fallax uit de wilde soorten S. bulbocastanum, S. fendleri en S. hougasii niet alleen op dezelfde locus liggen, maar bovendien hetzelfde werkingsspectrum hebben, wordt weinig additionele waarde verwacht van de strategie van stapelen van deze resistentie genen. De kweker zal dus op zoek moeten gaan naar nieuwe resistentiebronnen tegen de virulente nematoden populaties Col en/of Cbh, om een breed werkend resistente aardappel te ontwikkelen.

De in deze thesis gekarteerde resistentiegenen kunnen met behulp van moleculair merkers efficiënt in aardappel ingekruist worden. Bovendien zijn de resistentiegenen door de moleculaire merkers onderscheidbaar en daardoor wordt het mogelijk om resistentiegenen gericht te combineren. Dit zogenaamde stapelen van resistentiegenen zou kunnen leiden tot een bredere resistentie of een duurzamer resistentie.

Eén van de meest gebruikte woorden in het nawoord van proefschriften is "eindelijk". Als één proefschrift in aanmerking komt voor het gebruik van dit woord, dan is het dit proefschrift wel. Iets meer dan vier jaar na het beëindigen van het praktische werk is het eindelijk zover. Ik kan mijn promotieonderzoek afronden met dit proefschrift en de verdediging ervan. Of zoals veel vrienden en familie het noemen: mijn "studie" afronden.

Traditiegetrouw wordt in het nawoord iedereen bij name genoemd die een rol in het promotieonderzoek heeft gespeeld. Voor de goede orde: daar hoor jij ook bij, al wordt je niet bij name genoemd.

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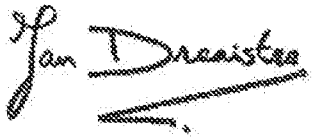
Mijn studenten Ad Vrolijk en Tae-Ho Park bedankt voor jullie inzet in mijn onderzoek. Zover ik kan overzien zijn jullie goed terecht gekomen.

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Zo, …

Nou is 't klaar!

Jan Draaistra werd geboren op 16 januari 1970 te Harkema. In 1988 werd het HAVO diploma behaald aan het Chr. Lyceum 'Oostergo' te Dokkum, waarna hij in 1993 de Agrarische Hogeschool Friesland te Leeuwarden succesvol afrondde. De goede resultaten in het laatste jaar werden beloond met de Boelstra-Olivier prijs. Hierna zette hij zijn studie voort aan de Landbouw Universiteit in Wageningen (LUW). Zijn afstudeeronderzoeken heeft hij uitgevoerd bij de vakgroep Nematologie en de vakgroep Erfelijkheidsleer. In 1996 studeerde hij af als plantenveredelaar. In april 1997 is hij in dienst getreden bij Hettema BV te Emmeloord en heeft vanaf 1 november 1997, gedetacheerd in Wageningen, voor de Stichting Bevordering Veredelingsonderzoek (SBV) zijn promotieonderzoek uitgevoerd. Hiervan staan de resultaten beschreven in dit proefschrift. Sinds 1 november 2001 is hij als Manager Breeding werkzaam bij Averis Seeds B.V. te Valthermond.

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Segregation analysis and RFLP mapping of the *R1* and *R3* alleles conferring race-specific resistance to *Phytophthora infestans* in progeny of dihaploid potato parents

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Abstract. *Phytophthora infestans* (Mont.) de Bary is the most important fungal pathogen of the potato (*Solanum tuberosum*). The introduction of major genes for resistance from the wild species *S. demissum* into potato cultivars is the earliest example of breeding for resistance using wild germplasm in this crop. Eleven resistance alleles (*R* genes) are known, differing in the recognition of corresponding avirulence alleles of the fungus. The number of *R* loci, their positions on the genetic map and the allelic relationships between different *R* variants are not known, except that the *R1* locus has been mapped to potato chromosome V. The objective of this work was the further genetic analysis of different *R* alleles in potato. Tetraploid potato cultivars carrying *R* alleles were reduced to the diploid level by inducing haploid parthenogenetic development of 2*n* female gametes. Of the 157 isolated primary dihaploids, 7 set seeds and carried the resistance alleles *R1*, *R3* and *R10* either individually or in combinations. Independent segregation of the dominant *R1* and *R3* alleles was demonstrated in two F_1 populations of crosses among a dihaploid clone carrying *R1* plus *R3* and susceptible pollinators. Distorted segregation in favour of susceptibility was found for the *R3* allele in 15 of 18 F_1 populations analysed, whereas the *R1* allele segregated with a 1:1 ratio as expected in five F_1 populations. The mode of inheritance of the *R10* allele could not be deduced as only very few F_1 hybrids bearing *R10* were obtained. Linkage analysis in two F_1 populations between *R1*, *R3* and RFLP markers of known position on the potato RFLP maps confirmed the position of the *R1* locus on chromosome V and localized the second locus, *R3*, to a distal position on chromdsome XI.

Key words: Potato – *Solanum tuberosum* – *Phytophthora infestans* – RFLP – *R* genes

Communicated by J. Schell

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Introduction

There are certain features which are common to the genes controlling race-specific resistances to fungal pathogens of crops like maize, barley and flax, that have been carefully characterised. These are: that (1) large numbers of dominant resistance alleles exist that differ in the recognition of the corresponding avirulence alleles of the fungus; and (2) groups of resistance alleles have been mapped to the same region on the genetic map, either as variants at the same or tightly linked loci (Saxena and Hooker 1968; Giese et al. 1981; Jahoor and Fischbeck 1987; Mayo and Sheperd 1980; Islam et al. 1989). For the host-pathogen interaction between the potato and *Phytophthora infestans* (Mont.) de Bary, 11 resistance or *R* genes have been identified so far, originating from the hexaploid wild species *S. demissum* (Black et al. 1953; Malcolmson and Black 1966; Ross 1986; Wastie 1991). The phenotype of the resistant alleles is a hypersensitive resistance reaction that takes place after infection by specific races of the fungus. When analysed, the *R* alleles have shown Mendelian inheritance as single dominant factors (Mastenbroek 1953; Malcolmson and Black 1966) and their relationship with the pathogen conforms to the gene-for-gene hypothesis of Flor (1942; 1971). Allelism among *R* alleles, linkage relationships, and map positions of the genetic loci involved are not known, with the exception of the *R1* locus which has been located on chromosome V of the potato RFLP map (Leonards-Schippers et al. 1992). It is, therefore, of interest to determine the genomic positions of other *R* loci relative to *R1* and to test whether or not *R* loci of potato are structured similarly to multiallelic resistance loci of other plant species.

One or more *R* alleles are present in many tetraploid potato varieties. They provided the first resistance phenotype against *P. infestans,* which causes the late blight of potato, one of the most destructive fungal diseases of this crop. They are the earliest examples of breeding for resistance in potato based on the introduction of germplasm from wild *Solanum* species, in the case

of the late blight of potato, from *S. demissum* (Ross 1986; Wastie 1991). Tetraploid potato varieties ($2n=4x=48$) known to carry *R* alleles were reduced, therefore, to the diploid level (Hermsen and Verdenius 1973) in order to obtain dihaploid parents ($2n=2x=24$) harbouring heterozygous *R* alleles which could be then used in crosses for the production of segregating F_1 populations. We have analysed such progeny segregating for specific *R* alleles and RFLP markers having known positions on the RFLP map of potato (Gebhardt et al. 1991; 1993). The analysis verified the map position of the *R1* locus (Leonards-Schippers et al. 1992) and revealed the existence and chromosomal position of a second locus, *R3*, in the potato genome.

Materials and methods

Origin of plant material. Ten cultivars reported to have one or more *R* alleles were selected from the Dutch variety list (Anonymous 1989). Dihaploids ($2n=2x=24$) were induced through prickle pollination using the diploid species, *S. phureja,* clones IVP 35 and 48 (Hermsen and Verdenius 1973) and IVP 101 (Hutten et al. 1990). The dihaploids obtained were tested with *P. infestans* race 0. The resistant dihaploids were grafted onto tomato root stock (cv. Virosa) for flower induction and pollination. The diploid clones 87-1017-5, 87-1024-2, 87-1029-31 and 87-1031-29 (Jacobsen et al. 1989) and J89-5002-6, J89-5002-18, J89-5040-1 and J89-5040-2 were used as pollinators. They were fertile and susceptible to *P. infestans* (*rr*).

Determination of fertility. Female fertility of the primary dihaploids and selected F_1 hybrids was determined after pollination with fertile diploids by berry set and seed set per berry. Male fertility was estimated after staining pollen with lactophenol acid fuchsin.

Inoculum. The *P. infestans* races 0, 1, 3.7, 4.10, 1.4.10, 1.3.4.7, 1.3.4.7.10, 1.3.4.10.11 and 3.4.7.8.10.11 were kindly supplied by L.J. Turkensteen (Research Institute for Plant Protection, IPO-DLO, Wageningen) and F. Govers (Department of Phytopathology, Wageningen Agricultural University). The isolates were maintained on tuber slices of cv. Bintje to ensure sufficient pathogenicity on foliage. The tuber slices were kept in moist trays in a dark climate chamber at 17° C. For preparation of the inoculum, fresh tuber slices were infected with a spore suspension. After 6 days, spores were collected by washing the slices with demineralised water. The concentration of the resulting spore suspension was adjusted to 10^4 conidia/ml.

Inoculation procedure. Plants (8- to 10-weeks-old) were tested using the detached leaflet technique (Toxopeus 1954; Mooi 1965; Umaerus 1969). For inoculation, fully expanded leaves were collected and fixed upright by their petioles in floral foam (Smithers-Oasis) saturated with water in a container with a transparent cover. A thin film of inoculum was sprayed on the lower surface of the leaves using a hand sprayer fitted with a fine nozzle. The containers were kept in a climate chamber at 17° C in 16 h light/8 h dark. The leaves were kept in ca. 100% relative humidity for 24 h, then ventilated to allow the leaves to dry. The disease assessment was carried out 5 days after inoculation. Plants were scored as: (1) resistant (R) when symptoms were absent or a hypersensitive reaction was visible; and (2) susceptible (S) when sporulating lesions developed. The resistance test was repeated twice. Parents, as well as defined genetic material resistant to specific races of *P. infestans* (C. Mastenbroek unpublished: tetraploid tester clones containing *R0; R1, R2, R3* and *R10,* and *R1 R3* loci), were used as controls in both tests.

RFLP analysis. Total genomic DNA was extracted from 0.3–0.4 g freeze-dried leaves and shoots of parental lines and F_1 hybrids a described previously (Gebhardt et al. 1989). DNA was purified by CsCl gradient centrifugation in all cases. Restriction digests, electrophoresis, electrotransfer to Nylon membranes (Amersham, Hybond N) and hybridization to RFLP marker probes were carried out according to Gebhardt et al. (1989). Linkage analysis among *R1, R3* and RFLP alleles was performed using computer programs based on the models and algorithms given by Ritter et al. (1990). Marker probes were selected according to their map position on an updated version of the potato RFLP map (Gebhardt et al. 1991; 1993). GP markers originated from genomic DNA of potato, and CP markers were derived from potato cDNA clones. The marker probe TG105 from tomato was provided by S.D. Tanksley (Cornell University, Ithaca, NY, USA). Small letters in parenthesis indicate that more than one RFLP locus is detected with the same probe.

Results

Isolation and characterisation of dihaploids

From ten cultivars bearing one or more *R* alleles, 157 parthenogenetic dihaploids were obtained. Some of them were malformed and of low vigour. All dihaploids were tested for resistance to *P. infestans* race 0, and 60 were resistant (Table 1). Assuming unbiased segregation of *R* alleles in dihaploids derived from tetraploid varieties, a segregation ratio of 1:1 (resistant versus susceptible) is expected for one *R* allele in the simplex constitution and of 5:1 in the duplex constitution. In varieties with two unlinked *R* genes both with one dominant allele, a 3:1 (both simplex) or an 11:1 (one simplex, one duplex) segregation ratio of resistant versus susceptible dihaploids is expected. Even higher frequencies of resistant dihaploids should be observed when more than two genes, each with one *R* allele, are present in the tetraploid variety.

The frequency of resistant dihaploids was much lower than expected in the dihaploid progeny of cvs. Morene, Eba and Radosa (Table 1). Also the five dihaploids extracted from cvs. Astarte and Atrela bearing two *R* alleles were, contrary to expectation, susceptible. On the

Table 1. Number of dihaploids isolated from ten cultivars and their reaction to *Phytophthora infestans* race 0

Cultivar	Number of dihaploids			
	Total	Test with race 0		χ^2 1:1
		Resistant	Susceptible	
R1				
Maritta	2	0	2	nt
Morene	39	2	37	31.40**
Saturna	6	4	2	nt
Total	47	6	41	26.06**
R3				
Cardinal	6	2	4	nt
Eba	30	8	22	6.53*
Radosa	28	6	22	9.14**
Total	64	16	48	16.00**
R1R3				
Astarte	3	0	3	nt
Atrela	2	0	2	nt
Total	5	0	5	nt
R1R3R10				
Hertha	22	20	2	nt
R1R2R3R10				
Escort	19	18	1	nt
Total	157	60	97	

nt, not tested
*, **, significant at $P=0.05$ and 0.01, respectively

Table 2. Segregation in the F_1 hybrids of resistant dihaploids crossed with susceptible diploids, after inoculation with *P. infestans* race 0.

Dihaploid resistant parent	F_1 population (Code)	Test with race 0 Number of plants			
		R	S	χ^2	
				1:1	3:1
R1					
J90-6026-4	J91-6146	10	15	1.00	
Her.44	J91-6166	1	3	nt	
R3					
CarEP.1	J91-6152...				
	J91-6155	2	34	28.44**	
CarEP.8	J91-6156...				
	J91-6162	12	128	96.11**	
RadEP.90	J91-6170	7	12	1.32	
J91-6162-27	J92-6454	20	26	0.78	
R1R3					
Esc.42	J91-6164	58	23	15.12**	0.50
	J91-6165	14	15	0.03	11.05**
R1R3R10R?					
Her.64	J91-6167...				
	J91-6169	39	52	nt	

nt, not tested
**, significant at $P=0.01$

other hand, most of the dihaploids derived from cvs. Escort and Hertha carrying four and three *R* alleles, respectively, were resistant. Due to the low numbers of dihaploids obtained, significant segregation ratios could not be determined for most of the sets of primary dihaploid progeny. Pooling the numbers of dihaploids of cultivars with only one *R* allele shows, nevertheless, a highly significant bias towards susceptibility (Table 1).

After grafting onto tomato rootstocks, 24 of the 60 resistant dihaploids flowered normally. More than 100 berries were set after pollination of about 400 flowers using different susceptible diploid pollinators. Berries as well as seeds were obtained from seven well-flowering dihaploids. They originated from cvs. Cardinal (CarEP.1; CarEP.8), Escort (Esc.42), Hertha (Her.44; Her.64), Radosa (RadEP.90) and Saturna (J90-6026-4). Seeds per berry varied among these dihaploid genotypes from 1.3 to 12.7. This indicates that 7 out of 157 primary dihaploids showed potential value for developing populations segregating for one or more *R* alleles. These seven dihaploids were tested with additional races of *P. infestans* to determine which of the *R* alleles were present. J90-6026-4 and Her.44 contained *R1*, CarEP.1, CarEP.8 and RadEP.90 contained *R3* and the dihaploids Esc.42 and Her.64 contained *R1R3* and *R1R3R10R?*, respectively (Table 2). The *R2* and *R10* alleles present in the cv. Escort were not transferred to Esc.42. A remarkable observation was that cv. Hertha was susceptible to race 1.3.4.7.10, whereas the dihaploid Her.64 was resistant, indicating the expression of a new *R* specificity in Her.64.

Seed set and segregation of F_1 plants

Both male and female fertility of the resistant primary dihaploids was low. Observations on F_1 hybrids in crosses among the dihaploids and susceptible pollinators indicated that flowering capacity and fertility increased. This was evident from the number of seeds per berry that increased from 1.3–12.7 in parental dihaploids to 28.4–77.3 in the F_1 hybrids.

Twenty sets of progeny were classified into susceptible (S) and resistant (R) plants using *P. infestans* race 0 as inoculum (Table 2). Nineteen sets resulted from crosses of the seven primary dihaploids with susceptible clones as pollinators. One set of progeny (J92-6454) originated from a cross between the F_1 hybrid J91-6162-27 (*R3r*) of progeny J91-6162 and the susceptible clone 87-1024-2 (*rr*).

The 1:1 ratio expected for the segregation of the *R1* and *R3* alleles present in the heterozygous state was found in populations J91-6146 (*R1*), J91-6170 and J92-6454 (*R3*). Population J91-6166 was too small to assess the segregation ratio. The sets of progeny resulting from crosses with dihaploids CarEP.1 and CarEP.8 of cv. Cardinal showed a highly distorted segregation ratio for *R3* in favour of susceptibility (Table 2). The two progeny sets J91-6164 and J91-6165 had the female parent Esc.42 in common, which carries *R1* plus *R3* and they differed only for the susceptible male parent. Assuming no link-

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Table 3. Segregation of *R* alleles in the F_1 hybrids of Esc.42 (*R1R3*) and Her.64 (*R1R3R10R?*) crossed with susceptible diploids

Populations	Number of plants with phenotype[a]						
	R1	R3	R1R3	R1R3R10	S	Not tested	χ^2 1:1:1:1
R1R3							
J91-6164	14	18	20	–	23	1	2.38
J91-6165	7	3	3	–	15	1	13.71**
R1R3R10R?							
J91-6167	19	–	2	1[b]	31	–	nt
J91-6168	13	–	1	1	15	–	nt
J91-6169	1	–	–	–	6	–	nt

nt, not tested
**, significant at $P=0.01$
[a] Other possible combinations between *R* alleles were not found
[b] This plant shows a resistance behaviour similar to that of the mother plant Her.64

age between *R1* and *R3*, a 3:1 segregation ratio (resistant versus susceptible) was expected and, indeed, found for progeny J91-6164. Progeny J91-6165 segregated, however, with a 1:1 ratio, compatible with *R1* and *R3* being closely linked in coupling phase (Table 2). Allelism between *R1* and *R3* was excluded because susceptible F_1 plants were found. The segregations observed in the progeny of Her.64 which, based on inoculation data, should have carried at least *R1*, *R3* and *R10*, did not fit the exceptation of at least two unlinked *R* loci, as too many susceptibles were found.

The populations J91-6164, J91-6165, J91-6167, J91-6168 and J91-6169 were further tested with a number of specific races (0, 1, 3.7, 4.10, 1.4.10, 1.3.4.7, 1.3.4.10.11, 1.3.4.7.10, and 3.4.7.8.10.11) in order to determine the presence of *R1*, *R3*, *R10* and combinations thereof in resistant F_1 individuals (Table 3). This analysis demonstrated again that *R1* and *R3* were unlinked in the populations J91-6164 and J91-6165. A regular segregation pattern of 1:1:1:1 for the phenotypes R1, R3, R1R3 and susceptibles (S) was found in progeny J91-6164 as expected for the segregation of two unlinked *R* loci, but not in progeny J91-6165. In this latter progeny, the segregation was distorted for *R3*, with an excess of susceptible plants (6 R3 versus 22 R1 or S, $\chi^2_{1:1}=9.14$, $P<0.01$) whereas the *R1* allele segregated as expected (10 R1 versus 18 R3 or S, $\chi^2_{1:1}=2.28$, $P>0.05$). The mode of inheritance of *R10* could not be deduced from populations J91-6167, J91-6168 and J91-6169, as only 2 out of 38 resistant plants had the R10 phenotype. The same was true for *R3* in these three crosses: only five plants expressed the *R3* allele (Table 3). The *R1* allele segregated, however, as expected in progeny J91-6167 (22 R1 versus 31 S, $\chi^2_{1:1}=1.18$, $P>0.2$) and J91-6168 (15 R1 versus 15 S, $\chi^2_{1:1}=0.0$, $P>0.8$). One plant of progeny J91-6167, J91-6167-35, showed the same resistance pattern as the mother plant Her.64 (Table 3).

RFLP mapping of R1 *and* R3

The progeny J91-6164 (Table 3), originating from the cross Esc.42 (*R1 r/R3 r*) × 87-1031-29 (*rr/rr*), was chosen in order to identify the positions of *R1* and *R3* on the RFLP linkage map of potato. Fifty-four plants (10 R1, 14 R3, 12 R1R3, 18 S) and the parents were analysed with 83 marker probes, chosen based on their positions on the 12 potato linkage groups, for cosegregation of RFLP alleles with the *R1* and the *R3* resistance alleles, respectively. Forty percent of all the markers tested were not informative, being either homozygous in the resistant parent Esc.42 or not polymorphic between both parents.

The map position of the *R1* locus on chromosome V, which was determined previously (Leonards-Schippers et al. 1992), was confirmed by testing the marker *GP21*, known to be closely linked to *R1* (2.5 cM). In progeny J91-6164, *R1* was linked (4.5 cM away) to the same marker locus (data not shown). The *R3* locus was identified at a distal position on chromosome XI (Fig. 1B). For comparison, an updated version of chromosome XI, as deduced from segregation data for 12 RFLP marker loci informative in the cross used for constructing our first RFLP map of potato (Gebhardt et al. 1991; 1993), is shown in Fig. 1A. The genetic distance between *R3* and the proximal group of three closely linked RFLP loci *GP250(a)*, *GP185* and *TG105(a)* was 7 cM. The most closely linked RFLP locus, *GP35(k)*, was not useful for the identification of the map position of *R3*, as the position of *GP35(k)* itself was unknown. The marker probe GP35 detects multiple loci in the potato genome (Gebhardt et al. 1989) only some of which show segregating RFLP alleles in any specific cross. *GP35(k)* was not detected in the cross used for constructing the chromosome XI map shown in Fig. 1A.

The position and the order of *R3* and of the RFLP loci linked to it were confirmed by segregation analysis using 41 plants and the parents of progeny J92-6454 (Table 2). The locus *R3* was found to be 2.4 cM (one recombinant) distal to *GP250(a)* and *TG105(a)* and only 5 cM distal to *GP38* (Fig. 1C), whereas in progeny J91-6164 the distance between *R3* and *GP38* was 29 cM, nearly 6 times larger. The recombination behaviour was, therefore, very different in populations J91-6164 and J92-6454. When compared with the recombination among the same marker loci in the RFLP mapping population shown in Fig. 1A, the cross J91-6164 showed increased, and the cross J92-6454 reduced recombination in the resistant parent.

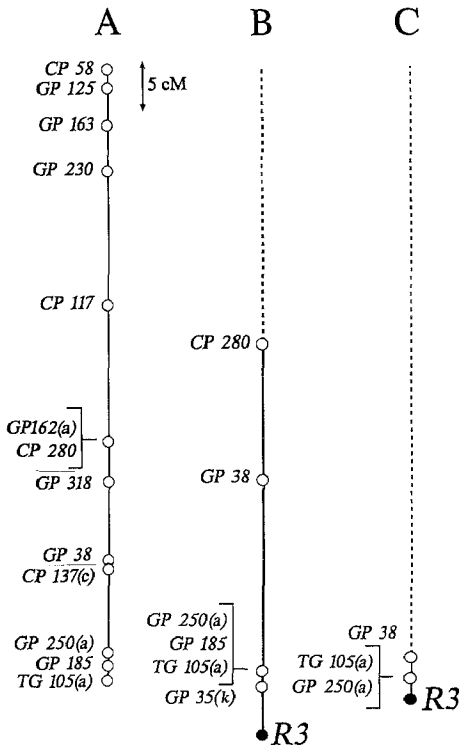


Fig. 1A–C. Map position of the *R3* locus on chromosome XI of potato. **A** Chromosome XI linkage map deduced from RFLPs segregating in the mapping population of Gebhardt et al. (1989; 1991). **B** Part of chromosome XI with the *R3* locus as derived from RFLP analysis in progeny J91-6164. **C** part of chromosome XI with the *R3* locus as derived from RFLP analysis in progeny J92-6454

Discussion

Only 7 out of 157 dihaploids extracted from tetraploid varieties expressing *R* alleles proved to be both fertile and resistant to specific races of *P. infestans*. The low efficiency was due to the low fertility and the preferential recovery of susceptible dihaploid plants. Low vigour and low fertility is an observation that holds for a large proportion of primary dihaploids of potato (Carroll and Low 1975; 1976; Ross 1986). It is explained as the phenotypic manifestation in the dihaploids of unfavourable recessive alleles present in the tetraploid. Such unfavourable alleles might also be particularly linked to the *R* alleles present in cultivated varieties which have been introduced from the wild species *S. demissum*. Although diluted by backcross breeding, considerable amounts of wild germplasm should still be present around the *R* loci (Stam and Zeven 1981). Linkage drag may be, therefore, the reason for the low frequency of resistant and fertile dihaploids recovered. Fertility improved in F_1 hybrids of the primary dihaploids crossed with fertile plants. This is in accordance with previous observations (Ross 1986 and references therein).

Segregation analysis of the alleles *R1* and *R3* in a series of F_1 populations originating from crosses of resistant with susceptible fertile diploids revealed that, in the majority of cases, the 1:1 ratio expected for the segregation of a single, dominant allele in heterozygous condition was highly distorted towards susceptibility. The *R3* allele was more affected than the *R1* allele: of the 18 populations segregating for *R3*, all but 3 (J91-6170, J91-6164, J92-6454; Table 2) exhibited the distortion, whereas in all 5 progeny segregating for *R1* (J91-6146, J91-6164, J91-6165, J91-6167, J91-6168) a normal 1:1 segregation ratio was found. Normal segregation of *R3* depended on the pollinator used, as becomes evident on comparing populations J91-6164 and J91-6165, which shared the female parent. Distorted segregation ratios are frequently observed in crosses of dihaploid potato lines (Gebhardt et al. 1991) and can be explained by postzygotic selection against unfavourable allelic combinations. In the crosses analysed, such unfavourable allelic combinations might have been present at the *R3* locus itself or at loci linked to *R3*.

RFLP analysis of the F_1 populations J91-6164 and J92-6454 confirmed the map position of *R1* and revealed that of the *R3* locus on the genetic map of potato. The location of *R1* on chromosome V (Leonards-Schippers et al. 1992) is supported by the close linkage of the *R1* allele segregating in progeny J91-6164 to the marker locus *GP21* on chromosome V. The *R3* locus was found at a distal position on chromosome XI linked to a proximal group of three marker loci *TG105(a)*, *GP185*, and *GP250(a)*. The tomato marker probe TG105 detects homoeologous RFLP loci on chromosome XI of *Lycopersicon* and *Solanum*, whose genomes are homoeologous (Tanksley et al. 1992).

In tomato, the marker locus *TG105* is closely linked (0.4 cM) to the *Fusarium oxysporum* resistance locus *I2* (Segal et al. 1992). The genetic distance of 0.4 cM was found to be equivalent to a physical distance of approximately 17 kb only (Segal et al. 1992). The *P. infestans* resistance locus *R3* in potato is linked to the same marker locus, however, depending on the progeny used for mapping, at genetic distances of 7 cM and 2.4 cM, respectively. The physical distance corresponding to this genetic distance is not known at present in the potato, which has about the same genome size as tomato (determined by flow cytometry, K.M. Blok, personal communication). It seems likely, however, that *R3* is also physically more distant from *TG105* in potato than *I2* in tomato. The large difference between the recombination frequencies separating the *R3* and *TG105* loci in two different crosses points to the fact that the correlation between genetic and physical distance is already highly variable within a single species. It depends not only on the position in the genome of the loci considered (Segal et al. 1992; Ganal et al. 1989) but also on the specific cross used in mapping experiments. Similar observations have been made in other plant species, for example in barley (Görg et al. 1993).

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R6 and *R7* alleles of potato conferring race-specific resistance to *Phytophthora infestans* (Mont.) de Bary identified genetic loci clustering with the *R3* locus on chromosome XI

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Abstract In potato, 11 resistance alleles (*R1–R11*) are known which confer race-specific resistance to the fungus *Phytophthora infestans*. *R1* has been mapped previously to potato chromosome V and *R3* to chromosome XI. Here we report on the localization of the *R6* and *R7* alleles on the genetic map of potato. Differential resistant strains of tetraploid *Solanum tuberosum*, clones Ma*R6* and Ma*R7*, were used as parental plants for the parthenogenetic induction and selection of diploid genotypes containing the *R6* or the *R7* resistance allele to *P. infestans*. One resistant dihaploid from Ma*R7* could be used directly as a parent to produce diploid F_1 progeny suitable for phenotypic and RFLP analysis. Ma*R6* did not produce useful dihaploids directly. After crossing Ma*R6* with a tetraploid susceptible genotype, resistant F_1 clones were selected. The resistant genotypes were then used as parents for the induction of dihaploids. Six dihaploids bearing *R6* were identified that could be crossed with a diploid susceptible genotype. Two diploid F_1 populations, segregating for *R6* and *R7*, respectively, were analysed with RFLP markers known to be linked with previously identified *R* genes. Markers linked with *R3* were found also to be linked with *R6* and *R7*. The resistance alleles *R6* and *R7* mapped to a similar distal position on chromosome XI as the *R3* allele.

Key words *Solanum tuberosum* · *P. infestans* · Resistance · Dihaploid induction · RFLP mapping

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Introduction

In potato, two types of resistance to *Phytophthora infestans* are known: one horizontal and the other vertical. The inheritance of horizontal resistance is based on polygenes (Umaerus et al. 1983; Wastie 1991; Leonards-Schippers et al. 1994),while the vertical resistance is controlled by major dominant genes (*R*-genes) (Mastenbroek 1953; Malcolmson and Black 1966). *R*-genes which confer race-specific resistance to *P. infestans* have been introduced from the wild species *Solanum demissum* into the cultivated tetraploid potato by repeated back crossing. Eleven *R*-genes are known (Malcolmson and Black 1966; Shaw 1991) and their isolation and characterization would help in understanding the mechanism of resistance to the fungus.

The mapping of *R*-genes is the first step towards molecular cloning either by transposon tagging or by map-based cloning.Two loci, named *R1* and *R3*, have recently been identified (Leonards-Schippers et al. 1992; El-Kharbotly et al. 1994) on chromosomes V and XI, respectively, using RFLP markers (Gebhardt et al. 1991; 1994). In the present paper we have determined the positions of the race-specific resistance alleles *R6* and *R7* in the potato genome by analyzing segregating populations at the diploid level. We find *R6* and *R7* to be located in the same chromosomal segment as the *R3* locus.

Materials and methods

Plant material

The tetraploid clones Ma*R6* and Ma*R7*, two differential tester clones carrying the *R6* and *R7* resistance alleles, respectively (C. Mastenbroek, unpublished), were used to develop diploid populations suitable for RFLP mapping. The clones were kindly supplied by Dr. L. J. Turkensteen (Research Institute for Plant Protection, IPO-DLO Wageningen, The Netherlands). From these clones dihaploids (2n=2x=24) were induced through prickle pollination using clones IVP 35 and 48 (Hermsen and Verdenius 1973) and IVP 101 (Hutten et al. 1994) of the diploid species *Solanum phureja*. The dihaploids obtained were tested with *P. infestans* race 0. Resistant dihaploids

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were grafted onto tomato root stocks of cv Virosa for flower induction and pollination, and used as seedstock in crosses with susceptible genotypes. In these crosses the fertile diploid clones 87-1024-2 (Jacobsen et al. 1989) and the amylose-free tetraploid clone J90-6001-3 (Flipse et al. 1996), which are both susceptible to *P. infestans* (*rr*), were used as pollen donors.

Screening for resistance

The resistance genotype of Ma*R6* and Ma*R7* was confirmed using different races of *P. infestans*. The same test was carried out for all susceptible pollinators and resistant F_1 hybrids. Races of *P. infestans* were kindly supplied by Dr. L. J. Turkensteen (Research Institute for Plant Protection, IPO-DLO Wageningen, The Netherlands) and Dr. F. Govers (Department of Phytopathology, Wageningen Agricultural University, The Netherlands). The preparation of *P. infestans* inoculum (races 0, 7, 2.7, 1.3.4.7 and 1.2.3.6.7) and the inoculation procedure were carried out according to El-Kharbotly et al. (1994).

Ploidy level determination

The ploidy level of the putative dihaploid seedlings was determined by counting the number of chloroplasts in their stomatal guard cells (Frandsen 1967).

RFLP analysis and mapping

RFLP analysis was carried out as described by El-Kharbotly et al. (1994) and Gebhardt et al. (1989). The linkage between the two resistance genes *R6* and *R7* and RFLP markers of chromosome XI was established on the basis of the fraction of recombinants recorded in the mapping populations. The linear order of loci was determined by three-point analysis. Closely linked loci were ordered by rejecting all alternate linear orders involving unlikely double-crossover events.

Results

Development of plant material for the localization of *R*-genes

Genotypes carrying a *R*-gene show an incompatible interaction resulting in hyper-sensitive resistance (R) when inoculated with *P. infestans* races carrying the corresponding avirulence gene. The same genotypes show a compatible interaction, leading to sporulating lesions (S), when inoculated with races lacking the corresponding avirulence gene. Clones not expressing *R*-genes are expected to be compatible (S) with all *P. infestans* races. The reaction pattern of clones Ma*R6* and Ma*R7* when inoculated with races 0, 7, 2.7, 1.3.4.7 and 1.2.3.6.7 was as expected, with the *R6* allele present in Ma*R6* and the *R7* allele in Ma*R7*. Pollinators used in dihaploid induction and crosses were susceptible to all races tested and did not, therefore, contain detectable *R*-genes (Table 1).

Because of its short flowering period, the production of dihaploids from clone Ma*R6* was difficult. From the mock pollination using *S. phureja*, three dihaploids were, nevertheless, isolated. All were resistant to *P. infestans* race 0 (Table 2), but either they did not flower at all, or else produced flower buds aborting before or after pollination. To overcome the problem of low dihaploid induction, the tetraploid clone Ma*R6* was crossed with the tetraploid susceptible clone J90-6001-3. Forty five F_1 plants (progeny J91-6145) were classified with respect to the *R6* genotype by testing them with *Phytophthora* race 0. A 1:1 or 5:1 (resistance versus susceptibility) segregation ratio was expected depending on the simplex (one *R6* allele) or duplex (two *R6* alleles) allelic constitution of Ma*R6*. A segregation of 39 resistant to 6 susceptible genotypes was found which fits a 5:1 ratio (χ^2=0.36, P>0.5), indicating that two *R6* alleles are present in Ma*R6* (Table 1). The induction of dihaploids was much easier in the J91-6145 population because of its long flowering period and high berry set. From five selected tetraploid genotypes resistant to *Phytophthora*, 42 parthenogenetic dihaploids were isolated (Table 2), of which 29 were resistant to race 0 (Table 2). They were grown to flowering and tested for seed set after pollination with the susceptible clone 87-1024-2. Six fertile genotypes were recovered (Table 2).

The dihaploids isolated from Ma*R7* flowered and produced enough seeds in 2x×2x crosses for segregation analysis. Out of six dihaploids, two showed resistance to race 0 (Table 2).

Segregation of *R* alleles in diploid F_1 families

Few of our resistant dihaploids set seeds when pollinated with line 87-1024-2, due to the well known problem of male and female partial sterility of the primary dihaploids of potato. Seven diploid F_1 families were obtained, six segregating for *R6* and one for *R7*, when screened using race 0. Four *R6*-segregating F_1 families, J92-6589, J92-6595, J92-6601 and BE93-4035, showed a segregation ratio not

Table 1 Resistance reaction of parental potato clones to five different races of *P. infestans*

Potato clone	Genotype	Inoculation with *P. infestans* race[a]				
		0	7	2.7	1.3.4.7	1.2.3.6.7
Ma*R6*	*R6R6r6r6*	R	R	R	R	S
Ma*R7*	*R7r7r7r7*	R	S	S	S	S
S. phureja	*rr*	S	S	S	S	S
J90-6001-3	*rrrr*	S	S	S	S	S
87-1024-2	*rr*	S	S	S	S	S

[a] R = resistant; S = susceptible

Table 2 Numbers of dihaploids isolated from tetraploid genotypes with defined *R*-genes and their reaction to *P. infestans* race 0

Potato clone	Number of dihaploids			Fertile dihaploids (codes)
	Total	Inoculation with race 0		
		R	S	
Ma*R6*	3	3	0	
J91-6145-2	6	4	2	J92-6435-1
J91-6145-4	6	5	1	
J91-6145-6	5	2	3	
J91-6145-7	17	12	5	J92-6441-1, 2 J92-6442-1,8,10
J91-6145-8	8	6	2	
Total	42	29	13	
Ma*R7*	6	2	4	BE93-4053-6

Table 3 Segregation in the progeny of crosses of resistant dihaploids (*Rr*) with the susceptible clone (*rr*) 87-1024-2. The inoculation was done with *P. infestans* race 0

Dihaploid resistant parent	Progeny-code	Inoculation with race 0		χ^2 1:1
		Number of plants		
		Resistant	Susceptible	
R6				
J92-6435-1	J92-6589	19	24	0.58
J92-6441-1	J92-6590	5	14	4.26*
J92-6441-2	J92-6592	1	2	nd
J92-6442-1	J92-6595	3	9	3.00
J92-6442-8	J92-6601	35	53	3.68
J92-6442-10	BE93-4035	4	7	0.82
R7				
BE93-4053-6	BE94-4101	39	64	6.07*

nd, not determined
* Significant at P=0.05

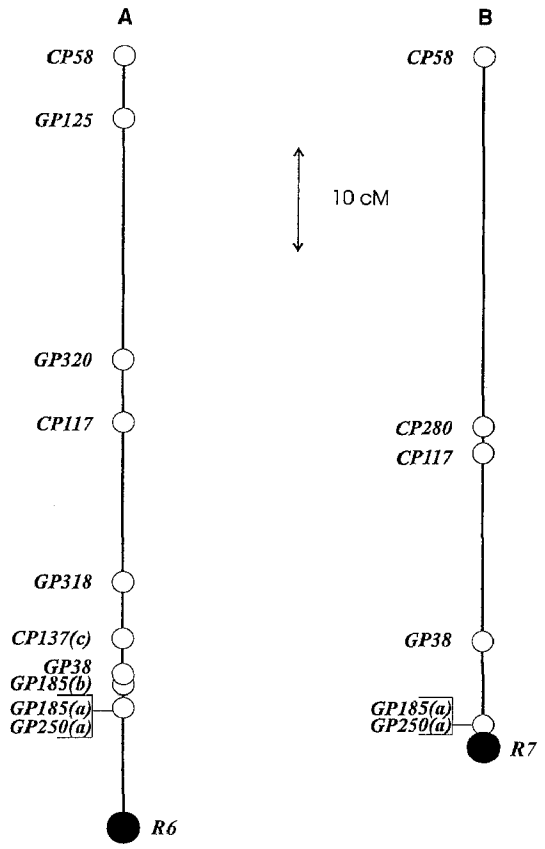


Fig. 1A, B Map position of the *R6* and *R7* resistance alleles on potato chromosome XI. **A** Linkage group derived from recombination in the *R6*-carrying parent J92-6442-8 of family J92-6601. **B** Linkage group derived from recombination in the *R7*-carrying parent BE93-4053-6 of family BE94-4101. GP markers are based on genomic DNA, CP markers on cDNA of potato. *Letters in parenthesis* indicate that more than one locus is detected by the same marker probe (Gebhardt et al. 1989)

significantly different from 1:1 at P=0.05. Family J92-6590 had an excess of susceptible genotypes (Table 3).

A 1:1 segregation ratio was expected also for the family BE94-4101 that segregated for the *R7* allele. In this population, however, a surplus of susceptible genotypes was observed (Table 3). The size of families J92-6601 and BE94-4101 was sufficient to assess the linkage between *R* genes and RFLP markers. The parental clones and the resistant genotypes of these two populations were tested with all available races of *P. infestans*. They showed the same phenotypes as the tetraploid clones *MaR6* and *MaR7* (Table 1). This test confirmed the presence of the *R6* allele in population J92-6601 and of the *R7* allele in BE94-4104.

Map positions of *R6* and *R7*

Parents and 85 plants of family J92-6601 and 96 plants of family BE94-4101 were analyzed with selected RFLP markers of known map position (Gebhardt et al. 1994). First preference was given to markers known to be linked to resistance genes in potato (Gebhardt 1994). Using RFLP marker probes GP185 and GP250, which detect closely linked RFLP loci [*GP185(a), GP185(b), GP250(a)*] on the distal end of chromosome XI in the vicinity of the *R3* locus (El-Kharbotly et al. 1994), linkage was evident to *R6* in family J92-6601 and to *R7* in family BE94-4101. Analysis with further RFLP markers of chromosome XI established the position of *R6* and *R7* distal to *GP185(a)* and *GP250(a)* (Fig. 1). *R7* was separated by one recombination event from marker loci *GP185(a)* and *GP250(a)*. The resistance phenotype of the plant with the recombinant chromosome was ambiguous and may have been misscored. If this plant is not considered, *R7* is linked without recombination to loci *GP185(a)* and *GP250(a)*. *R6* was separated from the same markers by ten recombination events. All ten recombinant plants had the *R6* allele, while the second class of recombinants bearing the susceptible *r6* allele was absent in family BE94-4101.

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Discussion

The *R6* and *R7* alleles inducing resistance against *P. infestans* races have been extracted from tetraploid potato genotypes and incorporated into diploid parental lines. As previously observed (El-Kharbotly et al. 1994), the recovery of fertile diploid lines was difficult. An additional complication was the short flowering period of the tetraploid clone Ma*R6*. This was avoided by selecting long-flowering tetraploids carrying the *R6* allele from the cross of Ma*R6* with a highly fertile tetraploid clone susceptible to *Phytophthora*. The cross provided new basic material for the development of diploid clones. Although four of five diploid F_1 families in which the *R6* allele segregated did not deviate significantly from the expected 1:1 segregation ratio (R vs S), the pooled data indicated an excess of susceptible genotypes. Significant deviations from canonical segregating ratios are frequent when considering the product of sexual reproduction in this vegetatively propagated species (Gebhardt et al. 1991). In spite of this, two F_1 families of sufficient size were obtained in which *R6* and *R7*, respectively, segregated as single dominant factors. Probing these families with RFLP markers known to be linked to other *R* genes of potato (Leonards-Schippers et al. 1992; El-Kharbotly et al. 1994) revealed that both *R6* and *R7* are located on the same segment of chromosome XI distal to the RFLP loci *GP185(a)* and *GP250(a)*, in a region containing the *R3* resistance locus. *R7* was closely linked with loci *GP185(a)* and *GP250(a)*, whereas *R6* was separated from the same marker loci by 12 cM. All recombinant plants in the interval *GP185(a)-R6* had the R6 phenotype. The absence in this population of the susceptible class of recombinants indicates that the allelic combination present in those genotypes may have been gametically or zygotically lethal.

R3, *R6* and *R7* were mapped in independent F_1 families relative to the same closely linked RFLP markers. Despite the fact that the linkage values between *R3*, *R6*, *R7* and these closely linked RFLP loci differed, the possibility exists that all three are alleles of the same locus. Alternatively, *R3*, *R6* and *R7* may be alleles at closely linked loci. Both possibilities exist in other systems of plant-fungus interactions which are compatible with the gene-for-gene hypothesis. Examples are flax and flax rust, barley and powdery mildew, and maize and its rust (reviewed in Pryor 1987). The clustering of *R3*, *R6* and *R7* is the first example for a similar genetic architecture of fungal resistance genes in the potato.

The *R3*, *R6*, *R7* gene cluster of potato occupies a chromosomal position homologous to that of the *Fusarium oxysporum* resistance locus *I2* which maps to the same segment of chromosome 11 of tomato (Segal et al. 1992). The RFLP locus *TG105(a)* is closely linked to *I2* in tomato (Segal et al. 1992) and to the RFLP loci *GP185(a)* and *GP250(a)* in potato (El-Kharbotly et al. 1994). In the crosses analyzed here, the marker TG105 was not informative. It is possible, that the genes encoding *I2* in tomato and *R3*, *R6*, *R7* in potato are members of an homologous gene family. DNA-sequence analysis of resistance genes cloned from distantly related plant species, such as *Arabidopsis* and tobacco, has revealed structural similarities between genes conferring resistance to very different pathogens, such as *Pseudomonas* and tobacco mosaic virus (Mindrinos et al. 1994; Whitham et al. 1994). Based on such structural similarities we have amplified related gene fragments of potato by PCR (unpublished results from the laboratory of C. Gebhardt). One of these fragments, when used as a marker probe, identifies an RFLP locus that co-segregates with the *R7* allele with the exception of only one plant which had an ambiguous resistance phenotype and may have been mis-scored (unpublished results). This opens up the possibility that resistance genes located in this syntenic chromosome segment of potato and tomato are structurally related to the resistance genes effective against bacterial and viral pathogens in *Arabidopsis* and tobacco, respectively. The homology between different fungal resistance genes in the closely related genera *Solanum* and *Lycopersicon* based on their precise chromosomal location, as reported here, might be even more significant than in the case of resistance genes effective against more different pathogens. In the future, molecular cloning of different members of resistance gene families may, therefore, provide a versatile and adaptive instrument for engineering disease resistance in crop plants.

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Genetic mapping from field tests of qualitative and quantitative resistance to *Phytophthora infestans* in a population derived from *Solanum tuberosum* and *Solanum berthaultii*

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Abstract

Under controlled field conditions, a *Solanum* backcross population segregated for resistance to *Phytophthora infestans*. The population ('BCT') had been derived previously by crossing the *Solanum tuberosum* dihaploid USW2230 × *Solanum berthaultii* PI473331 to obtain the hybrid M200-30, and then backcrossing the hybrid to the *S. tuberosum* dihaploid HH1-9. Resistance was assessed from analyses of epidemics in small plots of each individual genotype, and data were recorded as area under the disease progress curve (AUDPC). The parents of the original cross (USW2230 and a selection from PI473331) were not included in the test, but the hybrid was incompatible and HH1-9 was compatible with the tester strain of *P. infestans* (US-8 lineage). Somewhat more than half of the progeny also were incompatible with the tester strain, indicating the presence of an *R* gene. This gene segregated from the *S. berthaultii* parent and mapped 4.8 cm from the RFLP marker *TG63* on chromosome 10. We deduce that the *R* gene is not *R-1*, *R-2*, *R-3*, *R-6*, or *R-7* and is probably not *R-4*, *R-5*, or *R-10*. Among the remaining, compatible progeny, there was a wide range of quantitative resistance. All were more resistant than the susceptible cultivar Superior, and most individuals were much more resistant than the moderately resistant cultivar Kennebec. AUDPC values among the sub-population of compatible genotypes ranged from about 400 to 1500 units the first year and from 400 to 1760 units the second year. At least five quantitative trait loci (QTLs) were detected in this sub-population in both 1997 and 1998, including one detected through segregation of alleles from both the hybrid parent and the recurrent *S. tuberosum* parent. A model of main and epistatic effects explained 56% and 66% of the variation observed for quantitative resistance to late blight in 1997 and 1998, respectively. Several of the QTLs for late blight resistance were located in regions of the genome to which QTLs for late maturity have previously been mapped.

Abbreviations: AUDPC, area under the disease progress curve; BCB, backcross to *Solanum berthaultii*; BCT, backcross to *Solanum tuberosum*; QTL, quantitative trait locus

Introduction

Development of resistance to *Phytophthora infestans* (Mont.) de Bary, the causal organism of the late blight disease, is currently one of the highest priorities in potato (*Solanum tuberosum* L.) breeding programs worldwide. The urgency of obtaining resistant cultivars has intensified because of the recent spread of more virulent forms of the pathogen that were previously restricted to Mexico [16–18, 24].

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Two types of resistance to late blight have been described and used in potato breeding. One kind is conferred by a series of major, dominant genes that render the host incompatible with specific races of the pathogen. Eleven such '*R* genes' have been identified and are believed to have originated in *Solanum demissum* [33, 49, 55], which is native to Mexico. In addition, an unknown *R* gene has recently been detected *in S. tuberosum* ssp. *andigena* [47]. Unfortunately, resistance based on single *R* genes or combinations of *R* genes has not proved to be durable: new pathotypes quickly evolve that are virulent against the formerly resistant hosts [49]. A second type of resistance, which is quantitative in nature and race non-specific, is thought to be much more durable [43, 49].

These two types of resistance often co-exist in potato genotypes and segregating populations, and the effects of *R* genes can interfere with the detection of genes for quantitative resistance. To avoid such interference, some breeders even advocate elimination of *R* genes from breeding populations [8, 48, 55]. Whether one adopts this philosophy or takes the opposite approach of pyramiding *R* genes [36], it is helpful to know their location within the genome. To date, five of the eleven known *R* genes have been located on the potato map [10, 11, 29, 31].

The wild species *Solanum berthaultii* was one of many species examined by Van Soest et al. [54] as potential sources of late blight resistance. They found very high levels of what they considered to be race non-specific resistance in some accessions and suggested that this species could be useful in breeding programs. Colon and Budding [5] also found high levels of resistance in two *S. berthaultii* accessions. A study of the components of resistance in genotypes of one accession led to the conclusion that infection efficiency with *P. infestans* was very low, whereas it appeared that the second accession may have been incompatible with the race used [6]. Subsequent experiments [7], in which the *S. berthaultii* accessions were used as parents, provided further indications that one or more incompatibility genes were present.

The availability of two diploid backcross populations involving *S. tuberosum* and *S. berthaultii* and corresponding molecular genetic maps [2] enabled the present investigation of the inheritance of late blight resistance. One population, designated BCT, was obtained by backcrossing an interspecific F_1 hybrid clone to *S. tuberosum;* and the other population, designated BCB, was obtained by backcrossing the same clone to *S. berthaultii.* These populations have already been used to detect quantitative trait loci (QTLs) for trichome-mediated insect resistance [2], for ability to tuberize under long photoperiods [51], and for a number of other traits [41, 52, 56].

The purpose of our experiments was to test the hypothesis that resistance from *S. berthaultii* segregates in the backcross progenies (BCT and BCB). If such segregation exists, we wanted to determine the genetic control and architecture of resistance, to facilitate its use. Because our primary interest was to study quantitative rather than qualitative resistance of the progeny, a complex isolate of *P. infestans* compatible to seven potato *R* genes was used to determine resistance in the field. We report here the chromosomal location of an *R* gene derived from *S. berthaultii.* We also place QTLs for late blight resistance on the previously established map and compare them to QTLs for maturity.

Materials and methods

Plant material

The BCT family had been obtained [2] as follows. USW2230, a dihaploid *S. tuberosum* derived from the tetraploid cv. Saco, was crossed as a female parent to a *S. berthaultii* clone selected from PI473331 [2]. One of the resulting hybrids, M200-30, was used as a female and backcrossed to a different *S. tuberosum*, HH1-9. RFLP markers had previously been mapped in this population [2], and QTLs for tuberization and dormancy had also been located previously [51, 52]. As is typical of the offspring of heterozygous parents, BCT presented RFLP markers ('alleles') segregating from both the interspecific hybrid and the recurrent parent. Segregation from the hybrid parent was observed by noting the presence or absence of markers, designated 'B alleles', that originated in the *S. berthaultii* parent, against the background of *S. tuberosum.* Segregation from the recurrent *S. tuberosum* parent was observed by noting the presence or absence of markers, designated 'T^R alleles', that originated in HH1-9. The framework map based on segregation of B alleles consists of 81 RFLP markers at intervals ranging from 0.8 to 22.3 cM. The map based on segregation of T^R alleles is comprised of 35 markers, much less uniformly distributed.

The backcross to *S. berthaultii*, BCB, was obtained by using the same hybrid parent, M200-30, as a female

and B11B as the male parent. B11B was a different seedling selected from PI473331, the *S. berthaultii* accession from which the pollen parent for the original cross was taken. Plantlets of both backcross families had been maintained *in vitro* and tested free from major potato viruses and other pathogens.

Experimental design

Field experiments were conducted with 146 genotypes of BCT and 141 genotypes of BCB. The interspecific hybrid (M200-30) and both recurrent parents (HH1-9 and B11B) were included in the experiment; but the parents of the original cross were not included, nor were the other F_1 clones from which M200-30 had been selected.

In vitro plantlets were transplanted to the greenhouse on 20–22 May 1997. These were transferred to cold frames in mid-June, in preparation for transplanting during the final seven days of June 1997.

The experimental design consisted of three randomized complete blocks, with individual treatments of three plants of the same genotype in a plot. The between-plant spacing was ca. 20 cm and plots were separated by 1.6 m within a row. The between-row spacing was 0.9 m, and the plots were located such that each plot was immediately surrounded by an unplanted area. The cultivars Superior (early maturing, very susceptible to late blight) and Kennebec (medium late maturity, moderately resistant to late blight) were included as controls.

In 1998 a smaller field experiment was established, using the sub-population consisting of 60 of the BCT clones found to be compatible with the pathogen used to assess resistance in 1997. Methods were as in 1997, except that there was only one plant of each genotype per block. There were two blocks of 49 clones, and 11 clones were not replicated. Transplanting to the field was 6 July.

Phytophthora infestans

The resistance of individual progeny was assessed in response to *P. infestans* isolate US-940480, obtained from the *P. infestans* culture collection maintained at Cornell University. This isolate is a representative of the US-8 clonal lineage (*sensu* Goodwin *et al.* [19]) and was particularly aggressive on potatoes. In previous assessments, this isolate was demonstrated to be very complex, having compatibility to *R* genes *1*, *2*, *3*, *4*, *5*, *7*, and *10* [20]. It was incompatible with *R* genes *6*, *8*, and *11*. Additionally, it was incompatible with *R* genes from tomato, *Ph-1* and *Ph-2*.

Plots were inoculated on the evening of 14 August 1997. They were irrigated for several hours prior to inoculation so that soil and foliage were wet in the evening when inoculation occurred. Inoculum consisted of a sporangial suspension (250 sporangia per ml) applied with a hand-held sprayer, such that each plot received ca. 500 sporangia. Sporangia had been obtained from sporulating lesions on potato leaflets. After inoculation, plots were sprinkler irrigated 3–5 days per week in the evenings for 1–2 h (2 mm/h) until the end of the experiment (11 September), when ratings were discontinued. Irrigation frequency was lessened if natural rainfall kept the plants moist. In 1998 the same procedures were followed except that late blight ratings ended 22 September.

Disease assessment

The data analyzed were values for area under the disease progress curve (AUDPC) and were obtained from frequent assessments of disease severity in each individual plot. Disease severity was assessed visually using a modification [15] of a technique initially described by the British Mycological Society [1] except that standard diagrams [23] were used with very low levels of disease, and the scale was applied to the 3-plant plots in 1997 and to single-plant plots in 1998. AUDPC values were calculated as described by Shaner and Finney [40]. AUDPC has shown to be a robust statistic for assessing severity of plant disease epidemics [14]. It is a cumulative assessment of disease during the entire epidemic and can separate early severe epidemics from late severe epidemics.

On very resistant plants, late blight can be difficult to identify and quantify in the field. Necroses caused by *P. infestans* can be similar to necroses caused by other factors. Therefore, plants with very little necrosis (AUDPC values <75) were analyzed in two ways. Leaves with necrosis were brought to the laboratory, inoculated with the same isolate used in the field, and observed microscopically for sporulation after incubation for 24 h at 18 °C in 100% relative humidity. Very sparse sporulation of *P. infestans* was observed on many of these plants. Additionally, a subset of these plants was inoculated with an isolate of US-7 genotype (US-940486, compatible with tomato genes *Ph-1* and *Ph-2*).

Data analysis

Qualitative (compatible vs. incompatible) and quantitative (mean AUDPC) values of each genotype were used for mapping activities. The map position of the locus that conditioned qualitative resistance was determined by testing for co-segregation with RFLP loci on the BCT maps [2]; and three-point linkage analysis was used to identify the nearest markers using Map Manager QT [34], version 3.0b20.

The 63 genotypes with compatible responses in 1997 were treated as a separate sub-population to search for quantitative effects. Broad sense heritability (h^2) was calculated according to $h^2 = \sigma^2_{gen}/(\sigma^2_{gen} + \sigma^2_{env})$, where σ^2_{gen} is the variance among the genotypes and σ^2_{env} is the variance among the replications. Both main effects and interactive (epistatic) effects in the sub-population were examined, using the mean values of the three replications for each genotype. Main effects were examined through Map Manager, with simple interval mapping followed by composite interval mapping.

Composite interval mapping was carried out by a reiterative process as described by Hunt *et al.* [22]. For this process the marker closest to a given QTL was used as a cofactor in multifactor analysis, as follows. To scan each chromosome, all markers on the other chromosomes with LOD scores ≥ 1.5 were used as cofactors. If a marker on the chromosome being scanned had a LOD score ≥ 1.5 as shown by the multifactor analysis, then this marker was added to the list of cofactors used to scan successive chromosomes. If the LOD score of a marker decreased below 1.5, that marker was dropped from the list of cofactors for ensuing tests. If no marker on the chromosome was detected with a LOD ≥ 1.5 after the multifactor analysis, no marker from that chromosome was used. The multifactor analysis on all chromosomes was repeated until there was no further change in cofactors. For the surviving list of cofactors, a threshold of LOD = 2 was set for declaring an interval significant in a QTL model. Only QTLs that met these criteria for both 1997 and 1998 data are reported.

The large number of markers available for testing interactions meant that type I errors were likely unless stringent P values were chosen. With only 63 genotypes available in our sub-population of compatible genotypes, the adoption of low P values could cause us to overlook important interactions. We therefore elected other procedures to guard against type 1 errors. To search for interactions we first used qGene [38] to identify apparent QTLs that had significance levels of $P \leq 0.1$. Interactions among the QTLs so identified were tested through two-way ANOVA for significance at $P \leq 0.05$. When two or more significant interactions were found to be due to association with genomic regions on two chromosomes, all significant interactions were considered in a regression model. The interactions that had a P value > 0.05 in the simultaneous model were not considered as significant and were excluded. We also excluded interactions for which chi square analysis of the number of genotypes present in each of the four allelic categories indicated serious skewing ($P \leq 0.05$). Only epistatic interactions that satisfied all of the above conditions both in 1997 and in 1998 are reported. The analogous methodology was used to examine intra-locus interactions. All of the main effect and interactive QTLs significant by these criteria were entered into the model to calculate the percentage of the total phenotypic variance explained.

Although QTLs are not reported in this paper if they were not detected both years, those detected in only one year may be found through the Internet in SolGenes at http://probe.nalusda.gov:8000/plant/about solgenes.html

Results

Disease development

The recurrent *S. berthaultii* parent, the hybrid parent, and all of the individuals in the BCB population were incompatible with our isolate of *P. infestans* and remained healthy during the course of the 1997 experiment. By contrast, 63 of 146 BCT progeny were compatible with the complex isolate used. Many of the very resistant plants in both populations had small, nondescript necrotic spots. While *P. infestans* sporulated from many of these spots after incubation at 18 °C in 100% relative humidity, the sporulation was very limited and very different from the compatible response observed in other progeny. Laboratory inoculation of a small subset of the extremely resistant individuals with the isolate US-940486 of the US-7 lineage did not result in typical compatible lesions, whereas inoculations with this isolate onto tomatoes with the *Ph-1* gene resulted in compatibility.

Among the 63 compatible BCT progeny in 1997 there was a wide range of quantitative resistance, from AUDPC values of 424 to 1507 (Figure 1A). However the vast majority of these progeny were more resistant

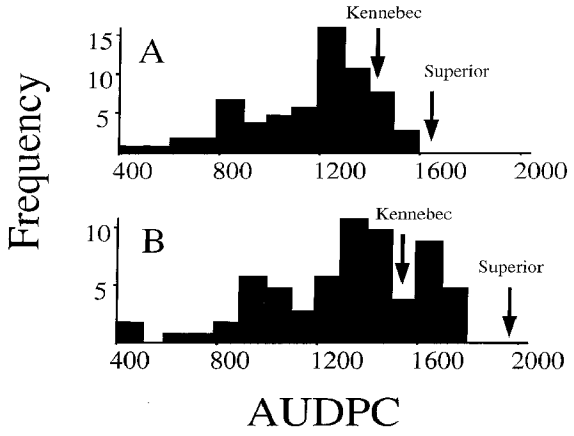


Figure 1. Frequency distribution of AUDPC (area under the disease progress curve) values within the sub-population of compatible genotypes, divided into 20 classes. A) 1997 values, 63 genotypes; B) 1998 values, 60 genotypes. The 83 incompatible genotypes (AUDPC values <75) from 1997 are not included. Arrows denote the AUDPC values for the control cultivars, Kennebec and Superior.

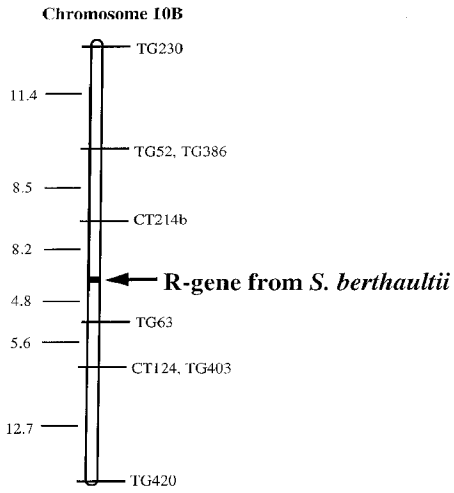


Figure 2. Map of chromosome 10, showing locus of the *R* gene derived from *Solanum berthaultii.* The map is based upon segregation from the hybrid parent (B alleles from *S. berthaultii*). Values on the left represent genetic distances (cM).

than either of the two commercial cultivars, Superior or Kennebec, which were included for comparison purposes. The cultivar Superior (very susceptible) was killed by late blight within three weeks of inoculation and had an AUDPC value of 1653. In contrast, cv. Kennebec (moderately resistant) succumbed more slowly and had an AUDPC value of 1406. HH1-9, the *S. tuberosum* recurrent parent used to make the BCT backcross, had an AUDPC value of 1170.

Epidemics in the small plots of individual progeny showing compatible reactions had typical logistic disease progress curves. Initial disease was visible within 4–6 days of inoculation and was generally well below 1% of the tissue affected. In some progeny, epidemics proceeded rapidly with many secondary cycles such that plants were killed within three weeks after initiation of the epidemic. Other progeny supported only slow epidemics with fewer secondary cycles, and with green foliage still remaining at the end of the assay. Plots of these progeny had low levels of AUDPC (400–800).

In 1997 the broad sense heritability for AUDPC within the sub-population of 63 compatible genotypes was 0.69. The coefficient of variation for the compatible clones, analyzed as a randomized complete block design in SAS using proc GLM, was 13.5% in 1997.

Results with the compatible sub-population of 60 BCT genotypes evaluated in 1998 were highly correlated with those obtained in 1997 ($r = 0.83$, $P<0.0001$) although the AUDPC values tended to be higher in the second year (Figure 1B). The AUDPC values for Superior and Kennebec were 1951 and 1501, respectively.

Genetic mapping

Loci associated with both qualitative and quantitative resistance were identified through genetic mapping. The extreme resistance phenotype (incompatibility) segregated most closely with an RFLP marker from *TG63*, originating in *S. berthaultii.* Of 83 incompatible progeny, 77 had a B allele present at *TG63*; and of 63 compatible progeny, only one had a B allele present at this marker. The X^2 for independent assortment of the marker and the compatibility reaction was 118.5 ($P<0.0001$). Three-point linkage analysis located a major gene effect consistent with the presence of an *R* gene on chromosome 10, 4.8 cm from RFLP marker *TG63*, and 8.2 cm from RFLP marker *CT214b* (Figure 2).

Within the sub-population of 63 compatible genotypes in BCT, differences in AUDPC values were associated both with segregation of B alleles (*S. berthaultii* source) from the hybrid parent and with segregation of T^R alleles from the recurrent *S. tuberosum* parent. Therefore maps based upon both types of segregation are presented (Figure 3).

We detected as main effects four QTLs in 1997 and three QTLs in 1998 (not shown). Two of these were common to the two years, and both were linked to

Table 1. Phenotypic effects of the QTLs detected (LOD ≥ 2.0) in both 1997 and 1998 for AUDPC through tests for direct (main) effects: segregation of B alleles from the hybrid parent, linked to *TG135* on chromosome 3 (a), and segregation of T^R alleles from the recurrent *S. tuberosum* parent, linked to *TG135* on chromosome 3 (b)

	LOD		Variance explained (%)		*n*		AUDPC	
	1997	1998	1997	1998	1997	1998	1997	1998
a) Presence[1] of B allele								
B+	2.34	2.97	10.9	13.9	28	23	1059 ± 52	1135 ± 69
B−					32	36	1228 ± 38	1370 ± 44
b) Presence[2] of T^R allele								
T^R+	2.34	4.17	13.1	23.9	36	36	1066 ± 46	1158 ± 53
T^R−					26	23	1251 ± 34	1467 ± 37

[1]Presence or absence of the B allele (at *TG449b* in 1997 and at *TG135* in 1998) is indicated by B+ or B−, respectively.
[2]Presence or absence of the T^R allele at *TG135* is indicated by T^R+ or T^R−, respectively.

marker *TG135* on chromosome 3 (Figure 3). In both years a QTL located in the interval between *TG449b* and *TG135* was detected through segregation of B alleles from the hybrid parent (Table 1a). This QTL was slightly closer to *TG449b* in 1997, and slightly closer to *TG135* in 1998 (Figure 3). The presence of B alleles in this interval was associated with a decrease of 207 AUDPC units, averaged over the two years. A second, complementary genetic effect was detected at *TG135* through segregation of an RFLP marker (T^R allele) from the *S. tuberosum* recurrent parent (Table 1b). A main-effect model, using the two QTLs associated with the *TG135* markers for B alleles and T^R alleles, accounted for 27% of the total phenotypic variation in AUDPC in 1997 and for 36% in 1998.

There was an interaction between the effects of the segregation of B alleles linked to *TG135* (Table 1a) and the segregation of T^R alleles linked to the same marker (Table 1b). Plants with both a B allele and a T^R allele present at *TG135* showed lower mean disease incidence than plants lacking one or both of these alleles (Table 2).

Inter-locus interactions

In addition to the interaction between B and T^R alleles at *TG135*, we detected four inter-locus interactions in both years of the study. Two cases of epistasis were defined by interactions between two QTLs detected through segregation of B alleles from the hybrid parent (Table 3; Figure 3); and two by segregation of B alleles at one locus and segregation of T^R alleles at a second locus (Table 4; Figure 3). A multiple-analysis model including the two main effects and the intra-locus interaction found near *TG135*, plus the four epistatic interactions observed in both years (Tables 3 and 4), explained 56% of the total phenotypic variance among the means of the 63 compatible genotypes in 1997 ($P<0.001$) and 66% among the 60 compatible genotypes in 1998 ($P<0.001$).

The pattern of all four epistatic interactions was consistent. Of the four possible combinations of alleles at the two QTLs in a given interaction, three combinations were associated with relatively high values of AUDPC. In contrast, the fourth combination of alleles was associated with an AUDPC value that was much lower (Tables 3 and 4). For example, in the interaction between the QTLs linked to *TG116* and *TG497*, the more resistant condition was observed when a B allele was present at both markers, whereas the absence of a B allele from either or both of these markers was associated with susceptibility (Table 3).

In each epistatic interaction, greater resistance was associated with the presence of alleles from *S. berthaultii* (Tables 3 and 4). For epistasis detected through the segregation of B alleles at one QTL and T^R alleles at the second QTL, the most resistant combination involved the presence of a T^R allele (along with the presence of a B allele) in one interaction and the absence of a T^R allele (along with the presence of a B allele) in the other interaction (Table 4).

Table 2. Apparent intra-locus interaction at *TG135*, chromosome 3, with comparison of AUDPC values for four genetic classes of BCT progeny

Presence [1] of B allele at *TG135*	Presence [1] of T^R at *TG135*	*P* value of interaction		Variance explained (%)		*n*		AUDPC[2]	
		1997	1998	1997	1998	1997	1998	1997	1998
		0.032	0.037	27.4	40.4				
B +	T^R +					15	15	918 a	969 a
B +	T^R −					10	8	1252 b	1446 b
B −	T^R +					21	21	1172 b	1294 b
B −	T^R −					16	15	1249 b	1478 b

[1]Presence or absence of the allele is indicated by + or −, respectively.
[2]Within each year, mean AUDPC values not followed by a common letter are significantly different from each other at $P \leq 0.01$.

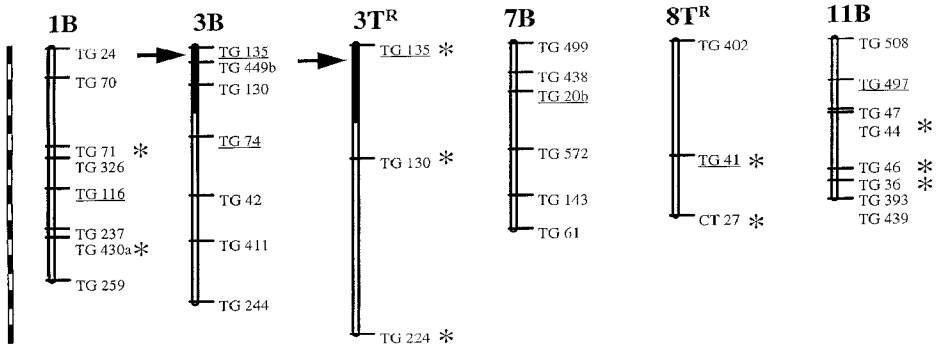


Figure 3. Location of QTLs associated with AUDPC values in the BCT sub-population of compatible clones. Arrows denote the estimated positions of QTLs detected by main-effect analysis. For main-effect QTLs, the black area on the chromosome shows the width of the peak before the likelihood decreases 10-fold. Underlined markers were detected through epistasis. The letter after the chromosome number indicates whether the map is based on segregation from the hybrid parent (*S. berthaultii* alleles, B) or from the recurrent parent (*S. tuberosum* alleles, T^R). Asterisks (*) mark QTLs detected previously for maturity [42, 51]. Each solid division on the scale at the left denotes a genetic distance of 5 cM.

Discussion

The progeny in BCT segregated for both qualitative and quantitative resistance to late blight. Segregation of extreme resistance was consistent with the origin of a major gene for incompatibility in *S. berthaultii*. This gene appears to have been heterozygous in the interspecific parent (M200-30), homozygous in the *S. berthaultii* backcross parent (B11B), and absent from the *S. tuberosum* backcross parent (HH1-9). Consequently the entire BCB progeny (M200-30 × B11B) was incompatible with the isolate of *P. infestans* used. The gene is located 4–5 cm from RFLP marker *TG63* on chromosome 10, a region that has been reported to contain other genes associated with resistance. The *St1.2.4* gene sequence, which contains base sequences characteristic of common resistance genes, maps to this same region of chromosome 10 [28], as does *Gro1.2* for resistance to *Globodera rostochiensis* [25, 27]. The gene segregating in our population has the characteristics of *R* genes (resistance is dominant and hypersensitivity is associated with the interaction), although its race-specificity has not yet been demonstrated. Quantitative resistance, due to genes of lesser effect, was also significant, with large differences between the most susceptible and the most resistant compatible progeny.

The fact that segregation of the *R* gene was skewed (ratio of compatible to incompatible 1:1.32) is consistent with the skewing observed at RFLP markers for B alleles on chromosome 10 in our map [2]. At *TG63* and *CT214b*, the markers adjacent to the *R* gene, the ratios for absence vs. presence of B alleles were 1:1.22 and 1:1.30, respectively. Skewed segregation in

Table 3. Interactions (epistasis) in BCT between two loci, both of which had B alleles segregating from the hybrid parent

First locus	Presence[1] of B at first locus	Second locus	Presence[1] of B at second locus	*P* value		*n*		AUDPC[2]	
				1997	1998	1997	1998	1997	1998
TG116(1)[3]		*TG497* (11)		0.016	0.007				
	B +		B +			14	14	917 a	1006 a
	B +		B −			17	16	1204 b	1392 b
	B −		B +			14	13	1234 b	1374 b
	B −		B −			15	15	1227 b	1355 b
TG135 (3)		*TG20b* (7)		0.014	0.038				
	B +		B +			18	17	958 a	1026 a
	B +		B −			7	6	1293 b	1444 b
	B −		B +			20	20	1199 b	1333 b
	B −		B −			17	16	1213 b	1419 b

[1]Presence or absence of the allele is indicated by B + or B −, respectively.
[2]Within a set of four values, means not followed by a common letter are significantly different from each other at $P < 0.01$; values followed by a common letter are not significantly different from each other at $P \leq 0.05$.
[3](Chromosome number).

tomato populations derived from crosses between *Lycopersicon esculentum* and *L. pennellii* was attributed to genes for sexual incompatibility, one of which is located on chromosome 10 [3, 13]. It is not known whether one or more similar genes for sexual incompatibility may account for the skewing observed in our population.

Comparison with known R *genes*

It is generally accepted [49, 55] that *S. demissum*, not *S. berthaultii*, was the source of the 11 identified *R* genes. It seems likely that the *R* gene detected in BCT has not been described previously. This resistance is not conferred by familiar alleles of *R-1*, *R-2*, *R-3*, *R-4*, *R-5*, *R-7*, or *R-10* in potatoes because the isolates of *P. infestans* used in our tests are compatible with these *R* genes. Additionally, *R-1* has been located on chromosome 5 [29]; *R-2* on chromosome 4 [31], and *R-3, R-6,* and *R-7* on chromosome 11 [10, 11]. The *R* gene from *S. berthaultii* identified in our study is on chromosome 10. By the process of elimination, we deduce that if the *R* gene from *S. berthaultii* that we have mapped to chromosome 10 is a previously described *R* gene from potato, then it could only be *R-8*, *R-9*, or *R-11* (or an allele not previously described at the locus of *R-4*, *R-5*, or *R-10*).

Given its map position, we considered the possibility that the *R* gene might be orthologous to *Ph-2*, previously described for resistance to *P. infestans* in tomato. Moreau *et al.* [37] placed *Ph-2* on the distal part of the long arm of chromosome 10 on the tomato map, which is highly co-linear with the potato map [45]. Although *TG63* was not included as a marker in the tomato experiments, the locus for the *R* gene segregating in our population is in the same region as the locus for *Ph-2*. However, inasmuch as our isolate of US-7 is compatible with *Ph-2*, and all of the highly resistant BCT plants tested were incompatible with US-7, the two factors clearly have different race specificities. Further studies are planned to determine the specificity and precise identification of this gene.

Quantitative resistance

The individual genetic effects described in this study are associated with AUDPC differences of from about 175 units to more than 400 units. Previous investigations [14, 15] concerning the value of genetic resistance in an integrated control strategy suggest that the depression of AUDPC by even 200 units has significant impact on epidemic development and the required schedule of fungicide application. Of the seven main or interactive effects contributing to resistance in BCT, five involved the segregation of alleles in the region of *TG135* on chromosome 3. In all cases, at this region or others implicated in the QTL analysis, introgressions from *S. berthaultii* were associated with reduced levels

Table 4. Interactions (epistasis) in BCT between one locus that had B alleles segregating from the hybrid parent with another locus that had T^R alleles segregating from the recurrent parent

First locus	Presence[1] of B at first locus	Second locus	Presence[1] of T^R at second locus	*P* value		*n*		AUDPC[2]	
				1997	1998	1997	1998	1997	1998
TG74(3)[3]		*TG41* (8)		0.048	0.005				
	B +		T^R+			10	10	1180 ab	1395 a
	B +		T^R−			21	18	1019 b	1024 b
	B −		T^R+			12	12	1157 ab	1348 a
	B −		T^R−			19	19	1256 a	1401 a
TG497 (11)		*TG135* (3)		0.021	0.006				
	B +		T^R+			20	20	975 a	1049 a
	B +		T^R−			8	7	1326 b	1565 b
	B −		T^R+			15	15	1182 b	1319 b
	B −		T^R−			17	16	1237 b	1425 b

[1]Presence or absence of the allele is indicated by + or −, respectively.
[2]Within a set of four values, means not followed by a common letter are significantly different from each other at $P < 0.01$; values followed by a common letter are not significantly different from each other at $P \leq 0.05$.
[3](Chromosome number).

of disease development. Intra- and inter-locus interactions demonstrated the additional importance of the allelic constitution of the recurrent *S. tuberosum* parent at *TG135* and *TG41*.

A possible explanation for the intra-locus interaction detected at *TG135* on chromosome 3 is that a single locus is involved. If so, the original *S. tuberosum* parent contributed a dominant allele for susceptibility, and both the recurrent *S. tuberosum* parent and the hybrid parent were heterozygous for the trait. However, the segregation of B alleles at *TG135* was epistatic with a QTL on chromosome 7, whereas the segregation of T^R alleles at *TG135* was epistatic with a QTL on chromosome 11. The difference in epistasis would not be expected if there is a single resistance locus linked to *TG135*, unless certain allele combinations at the locus interact with the QTL on chromosome 7, and others with the QTL on chromosome 11. Finer mapping with larger populations may help resolve whether one or two resistance loci are involved. Finer mapping aimed at isolating these factors may also help determine whether the QTL linked to *TG74* on chromosome 3 is distinct from the one near *TG135*.

Comparison with other reports of QTLs for late blight

Leonards-Schippers *et al.* [30] reported 11 QTLs on nine chromosomes for late blight resistance assessed through a leaf disc assay. Their population was obtained by backcrossing a hybrid between *S. tuberosum* and *S. spegazzinii* to a diploid line of *S. tuberosum*. As far as can be determined through an examination of common markers, QTLs that they reported to be present on chromosomes 3, 7, and 11 could be congruent to ones that we detected. They also found QTLs on chromosome 5 near a cluster of genes for resistance that includes *R-1* gene for late blight [29] and genes for resistance to insects [2], potato virus X [9], and cyst nematodes [26, 39]. In 1997 we detected a QTL in this region through epistasis, but the interaction was not significant in 1998 (data not shown).

Meyer *et al.* [35] identified a multi-allelic locus for late blight resistance in tetraploid potato. This locus was at the proximal end of chromosome 8, in the same region to which a single resistance gene analogue had been mapped by Leister *et al.* [28]. We detected a resistance QTL at *TG41* through segregation of T^R alleles in the same general region of chromosome 8.

Association of quantitative resistance with physiological maturity

It has long been observed that quantitative resistance to late blight tends to be associated with late maturity [8, 44, 46, 50]. There are several possible explanations for the association. It may be that genes for late maturity incidentally accompanied genes for late blight resistance when selections were made from *S. demis-*

sum [55], or that there has been a stronger selection for resistance in late maturing cultivars [8, 44]. A third possibility is that one or more of the polygenes contributing to late maturity has a direct or pleiotropic effect on late blight resistance.

There is evidence that the potato plant becomes more susceptible to infection as the foliage matures [21, 32]; and when the same cultivar was planted on successive dates during May and June, and plots were inoculated with *P. infestans* during late July or early August, the earliest plantings were most susceptible [15]. It follows that, at a given evaluation date, earlier maturing genotypes that do not otherwise differ in resistance may be more susceptible than later maturing genotypes planted at the same time. Colon [4] found that resistance to late blight was conditioned by photoperiod, which also affects maturity [12]. An association between QTLs for late maturity and resistance to late blight has also been observed [53]. Thus we were interested to determine in our population the extent to which QTLs for late blight resistance could be differentiated from QTLs for maturity.

Of the five chromosomes on which we detected QTLs for late blight resistance, four also carried QTLs governing tuberization and maturity, coincident with or in close positioning to the QTLs for late blight resistance (Figure 3). For example, the chromosome region detected for late blight resistance near *TG135* on chromosome 3 in the same population also contains a QTL influencing tuberization and maturity, identified previously by Van den Berg *et al.* [51]. Although the segregation of B alleles at *TG135* had no detectable effect on maturity, the presence of a T^R allele at *TG135* was associated both with later maturity [51] and greater resistance to late blight.

QTLs associated with resistance and maturity [51] have now been detected through segregation of T^R alleles near *TG41* and through segregation of B alleles near *TG116* [51]. In addition to the QTLs reported by Van den Berg *et al.* [51], we have found QTLs for maturity as measured by days from planting to tuber formation [42]. One of these QTLs is coincident with or close to the QTL for late blight resistance linked to *TG497* (chromosome 11, B alleles).

Where there was commonality of QTLs, the presence of an allele associated with earlier maturity was also associated with greater susceptibility to disease. At the present level of map resolution, we cannot distinguish whether these QTLs for resistance to late blight are pleiotropic with late maturity or may be closely linked. Either hypothesis is consistent with the common observation that selection for resistance tends to result in late maturity. The fact that other QTLs for maturity and for late blight resistance are *not* congruent is consistent with the observation that substantial differences in resistance exist within each maturity class: some early cultivars are less susceptible than other early cultivars, and even some very late cultivars are highly susceptible (e.g., 'Green Mountain').

Our study illustrates how genetic mapping of several quantitative traits in a common segregating population can be helpful to the breeder. If pleiotropy or linkage exists between one or more of the genes for an undesirable trait such as late maturity and one or more of the genes for late blight resistance, knowledge of which QTLs for the traits coincide, and which do not, should help breeders avoid the undesirable effects. Such information would also be important if genes for quantitative resistance to late blight are to be cloned.

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Molecular Cloning and Expression Analysis of a FT Homologous Gene from *Solanum tuberosum*

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Abstract

A homologue of flowering locus T gene, designated *StFT*, was isolated from *Solanum tuberosum* by reverse transcriptase-polymerase chain reaction (accession no. GU223211). The DNA sequence of *StFT* was 1 626 bp long and contained four exons and three introns. The open reading frame of the gene was 522 bp long and encoded a putative protein of 173 amino acids with a molecular weight of 19.75 kD and a theoretical *pI* of 7.76. StFT protein had a conserved PBP domain and a higher degree of identity with FT homologous members from other species. Analysis on the mRNA levels of *StFT* showed that it was highly expressed in leaves, apical buds, flowers, and swelling stolons. Further analysis indicated that its expression was regulated by *CONSTANS* gene in *StCOL*-antisense transgenic potato plants.

Key words: cloning, expression, *StFT* gene, *Solanum tuberosum*

INTRODUCTION

Fluctuations in day length determine the time to flower in many plants. During the growth of plants, day-length duration is sensed by light receptor phytochrome B (Schepens *et al.* 2004), which activates the abundance of CONSTANS (CO) protein with cryptochromes, and transforms the signal into a systemic signal and induces flower development (Valverde *et al.* 2004).

Flowering locus T (FT), a 20-kD protein, is a member of the small CETS protein family (Kardailsky *et al.* 1999; Kobayashi *et al.* 1999). FT acts as a potent promoter of flowering in long-day plants (LDP) such as *Arabidopsis thaliana*, short-day plants (SDP) such as rice and *Pharbitis nil*, and day-neutral plants (DNP) such as tomato (Turek *et al.* 2008). In *Arabidopsis*, the expression of FT in cotyledons and leaves is induced by the B-box zinc-finger protein CO (Suárez-López *et al.* 2001; Valverde *et al.* 2004). Then FT protein interacts with a bZIP transcription factor FD and activates transcription of meristem identity genes such as *APETALA1* (*API*), causing flower bud formation (Abe *et al.* 2005; Notaguchi *et al.* 2008). Up to date, *FT* gene has been cloned in *Arabidopsis* (Kobayashi *et al.* 1999), rice (Kojima *et al.* 2002), wheat (Yan *et al.* 2006), tomato (Lifschitz *et al.* 2006), Japanese morning glory (Hayama *et al.* 2007), grapevine (Carmona *et al.* 2007), cucurbit (Lin *et al.* 2007), barley (Hemming *et al.* 2008), *Chenopodium rubrum* (Chäb *et al.* 2008), *Sinapis alba* (Aloia *et al.* 2009), orchid (Hou and Yang 2009), apple (Kotoda *et al.* 2010), sunflower (Blackman *et al.* 2010), etc. However, the cloning of *FT* gene in potato has not been reported.

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In potato, flowering and tuberizing signals might be similar (Rodríguez-Falcón *et al.* 2006; Jackson 2009). Martínez-García (2002) reported that constitutive overexpression of *AtCO* in transgenic potato plants resulted in an impairment of tuberization under short days. Sarkar (2008) suggested that *AtCO* acts together with *PHYB* and produces the systemic signals consisting of inductive as well as inhibitory signals. A relative balance between these two opposite signals determines flowering or tuberization. As one of the main targets of CO in flowering regulatory pathway, whether FT involves in tuberization regulation in potato? Cloning *FT* gene from potato might be put forward for understanding CO mediated signal pathway of tuberization regulation. In this paper, we reported the results on the cloning of *StFT* cDNA from potato leaf using reverse transcriptase-polymerase chain reaction (RT-PCR) technique and its expression pattern in different tissues of donor plants and *StCOL*-antisense transgenic potato plants.

MATERIALS AND METHODS

Plant materials

In vitro plantlets of the potato cultivar Désirée (*Solanum tuberosum* L.) and *StCOL*-antisense transgenic Désirée (constructed by our laboratory) were transplanted to pots at the beginning of March 2009 and grown in a greenhouse at 23-25°C under a 10-12 h photoperiod, and watered every two days. Two months later, leaves of Désirée plants were collected for cloning of *StFT* cDNA. About three months later, different organs of the two genotypes above were harvested for expression analysis of *StFT*.

Isolation of full-length cDNA and genomic DNA of StFT

Total RNA was extracted from 0.1 g of fresh leaves with total RNA isolation reagent (Tiangen, China) following the manufacturer's instructions. First-strand cDNA was synthesized with M-MLV reverse transcriptase from Promega (USA) according to the manufacturer's instructions.

To clone the conserved region of *StFT* cDNA, a pair of primers, F1 (5′-TTCTACACTCTGGTCATGGTG-3′) and F2 (5′-CGCCACCCTGGAGCATACAT-3′) were designed according to the conserved regions of *FT* genes from other plants using the DNAssist 2.0 software. The PCR product was firstly incubated at 94°C for 5 min, and then incubated by a stepped program (94°C for 30 s, 51°C for 30 s, 72°C for 30 s) for 30 cycles, and by an extension at 72°C for 10 min.

To obtain 5′ end sequence, a primer F3 (5′-ATGCCTAGAGTTGATCCATTGATAG-3′) was designed according to 5′ end sequence of *FT* from *Ipomoea nil* (accession no. EU178860). The PCR was carried out using F3 and F2, according to the following program: 94°C for 4 min, followed by 30 cycles (94°C for 30 s, 55°C for 40 s, 72°C for 40 s) and by extension at 72°C for 10 min.

To obtain 3′ end sequence, a primer F4 (5′-TCATCGTCTCCGGCCTCC-3′) was designed according to 3′ end sequence of *FT* from *Ipomoea nil* (accession no. EU178860). Primary amplification was carried out using F1 and F4, under the following PCR condition: 94°C for 4 min, followed by 30 cycles (94°C for 30 s, 52°C for 30 s, 72°C for 30 s) and by extension at 72°C for 10 min. An aliquot of 1 μL (1:20 diluted) primary amplification products was used for 3′ end nested amplification under the same PCR condition using gene specific primers F5 (5′-CCGGTCAC AGATATCCCAGC-3′) designed according to the conserved region of *StFT* cDNA and F4.

The full length of the gene cDNA was amplified with gene-specific primers F3 and F4 after assembling the full-length sequence of *StFT*. PCR conditions were: 94°C for 4 min, followed by 30 cycles (94°C for 30 s, 53°C for 40 s, 72°C for 40 s) and with a final extension step at 72°C for 10 min.

The genomic DNA sequence of *StFT* was obtained from direct PCR of genomic DNA using the specific primers F3 and F4. PCR was carried out under the following conditions: 95°C for 5 min, then incubated by a stepped program (94°C, 40 s; 53°C, 40 s; 72°C, 2 min) for 30 cycles, and an extension at 72°C for 10 min.

All PCR products were separated on 1% agarose gels and target DNA bands were recovered by gel extraction and cloned into pMD18-T vector (TaKaRa Biotech, Dalian, China), and finally transformed into competent

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cells of *E. coli* strain DH5α. White clones were checked by PCR, and the positive clones were sequenced (Invitrogen Biotech, Shanghai, China).

Sequence analysis

Sequencing data accumulation, processing and sequence alignment analysis were performed using DNAMAN. BLASTp was performed on http://www.ncbi.nlm.nih.gov/blast. The open reading frame (ORF) of the sequence was predicted using GenBank ORFfinder (http://www.ncbi.nih.gov/gorf/gorf.html). The protein primary structure (theoretical isoelectric point and mass values) and domains analysis were predicted respectively using ProtParam (http://www.expasy.org/tools/protparam.html) and Smart (http://smart.embl-heidelberg.de/smart/) programs. The tertiary structure of the protein was predicted using online Swiss Model server (http://swissmodel.expasy.org/). The phylogenetic tree was constructed with DNAMAN-TreeView.

Expression analysis of *StFT* in different tissues

For the organ expression studies, white fibrous roots, stems, mature leaves, apical buds, flowers, initial stolons, stolons, swollen stolons (that is stolons with enlarged tips), little tubers, and mature tubers were harvested from both Désirée and *StCOL*-antisense transgenic Désirée plants cultivated in a greenhouse. The materials were frozen immediately in liquid nitrogen and stored at -70°C. A fragment of the potato GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was amplified as a positive control using the primer GAPDHS (5′-TCAACGAGAATGAATACAAGCCA-3′) and GAPDHA (5′-TCGACAACAGAAACATCAGCAGT-3′). The initial denaturation step of 4 min at 94°C was followed by 40 s at 94°C, 40 s at 54°C and 40 s at 72°C for 26 cycles, and the final extension was carried out for 10 min at 72°C. The corresponding amount of cDNA was used as template among samples with *StFT* specific primers F2 and F3 using the same reaction conditions described above, but with only 26 cycles.

RESULTS

Cloning of *StFT* gene

The cloning of *StFT* was stated by amplifying an approximate 230 bp fragment of the conserved region, followed by generation of a 270 bp 3′ fragment and a 400 bp 5′ fragment. The full length of the *StFT* cDNA was obtained using a pair of specific primers designed according to the assembled sequence of the gene (accession no. GU223211). The cDNA contained an open reading frame of 522 bp coding a protein of 173 amino acids, corresponding to a 19.75 kD polypeptide with an isoelectric point of 7.76. Its corresponding genomic sequence was 1 626 bp long and consisted of four exons and three introns (first exon 1-180; first intron 181-491; second exon 492-551; second intron 552-1 145; third exon 1 146-1 186; third intron 1 187-1 481; four exon 1 482-1 626) (Fig.1). This structure was similar to those of other plants, even though there was a big difference in the length of the introns among these species (Table 1).

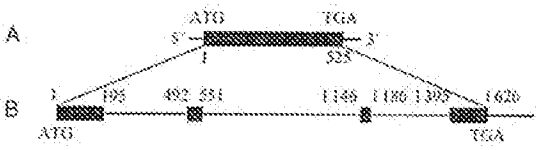


Fig. 1 Schematic representation of *StFT* gene. A, schematic diagram of the *StFT* cDNA ORF (black boxes) with 3′ and 5′ UTR (thin lines). B, exon-intron organization of the *StFT* gene with location of introns (thin lines) and exons (black boxes). The number 1 indicates the location of the translational start codon as determined. Numbers delineate addresses in nucleic acid sequences.

Table 1 Organization of introns and exons in *FT* genomic sequence from different plants

Name of organisms	Accession no.	No. of introns	Length (bp)	No. of exons	Length (bp)	No. of amino acid residues
Arabidopsis thaliana	NC003070	3	814, 712, 123	4	201, 61, 40, 223	173
Solanum tuberosum	GU223211	3	319, 593, 294	4	195, 59, 40, 231	173
Solanum lycopersicum	AY186737	3	771, 518, 256	4	195, 61, 40, 124	140
Malus × domestica	DQ535887	3	105, 190, 801	4	198, 61, 40, 223	174
Brassica napus	FJ848914	3	809, 342, 75	4	201, 61, 40, 223	175
Vitis labrusca × Vitis vinifera	EF203919	3	137, 907, 679	4	198, 61, 40, 223	174
Phyllostachys meyeri	AB498761	3	168, 125, 90	4	201, 61, 40, 232	178

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Sequence analysis of the StFT protein

An alignment of the predicted amino acid sequences of *S. tuberosum* StFT, *A. thaliana* AtFT (accession no. NP176726), *Ci. unshiu* CiFT2 (accession no. BAF96644), *C. sativus* CsFT (accession no. BAH28253), *M. domestica* MdFT (accession no. ACL98164), and *I. nil* InFT (accession no. ABW73563) was conducted using the DNAMAN program. The result showed that StFT protein had a higher identity with MdFT (80%), CsFT (79%), InFT (77%), CiFT2 (75%), and AtFT (74%) at the overall amino acid level, and especially in their N-and C-terminal regions (Fig.2).

Online Smart Server analysis found that in N-terminal at position 18-163, there was a conserved domain named PBP, a specific phosphatidylethanolamine-binding protein domain (Kardailsky *et al.* 1999), which was similar to the *Arabidopsis* FT protein structure. The tertiary structure of the StFT protein was very similar to *Arabidopsis* FT, too (Fig.3).

A phylogenetic tree was constructed using the sequences of FT/FT-like proteins downloaded from different species from GenBank (Fig.4). The phylogenetic tree analysis showed that all members could be

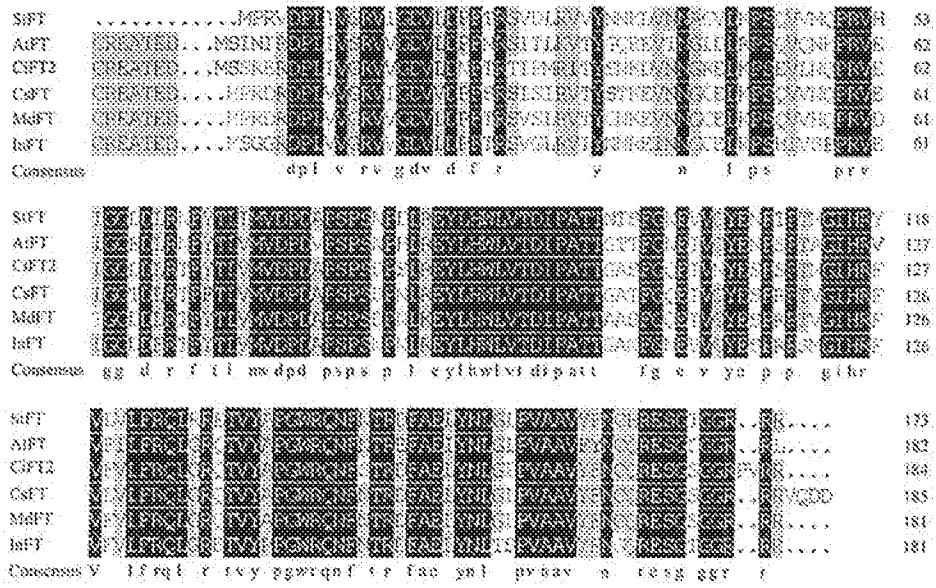


Fig. 2 Alignment of the amino acid sequence of StFT (accession no. ADA77529) with that of other homologous AtFT (accession no. NP176726), CiFT2 (accession no. BAF96644), CsFT (accession no. BAH28253), MdFT (accession no. ACL98164), InFT (accession no. ABW73563). The identical amino acids are shaded in black and the conserved amino acids are in gray.

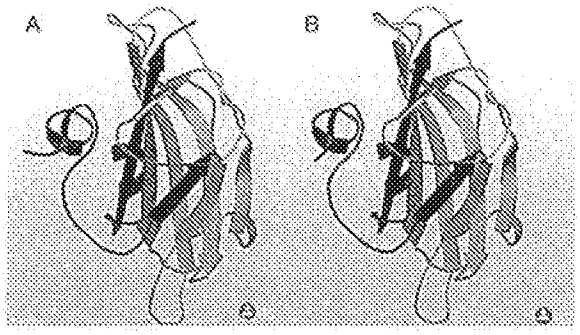


Fig. 3 Tertiary structure model of AtFT1 (A) and StFT (B).

divided into four divergent groups and that the StFT protein was clustered to InFT and had a high homology with CiFT2 from *Ci. unshiu* and AtFT from *A. thaliana*.

Expression of *StFT* gene in different tissues

The results on expression analysis of *StFT* gene in different tissues were presented in Fig.5. *StFT* was expressed in all organs and the level of transcript varied among the different tissues. High level of *StFT* expres-

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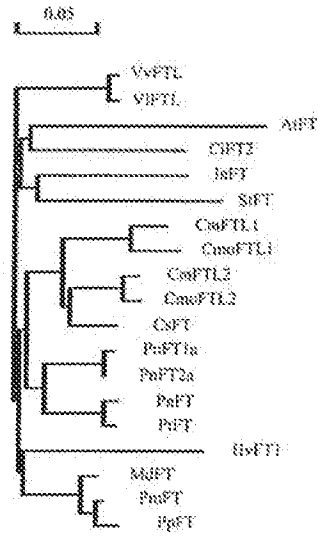


Fig. 4 Phylogenetic analysis of StFT and the FT or FT-like proteins from different plant species. The tree is displayed as a phylogram in which branch lengths are proportional to distance. The proteins are as follows: AtFT (NP176726), CiFT2 (BAF96644), CmFTL1 (AB194605), CmFTL2 (AB194606), CmoFTL1 (ABR20498), CmoFTL2 (ABR20499), CsFT (BAH28253), HvFT1 (AAZ38709), IaFT (ABW73563), MdFT (ACL98164), PmFT (CAQ16124), PnFT1a (BAD01612), PnFT2a (BAD01561), PaFT (BAD02371), PpFT (ACH73165), PtFT (ABD52003), VlFTL (ABN46891), VvFTL (ABN46890), and StFT (ADA77529).

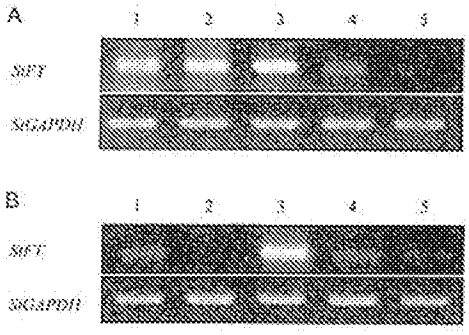


Fig. 5 Expression analysis of *StFT* in different tissues. A: 1, flowers; 2, leaves; 3, apical buds; 4, stems; 5, roots. B: 1, initial stolons; 2, stolons; 3, swelling stolons; 4, small tubers; 5, mature tubers.

sion was observed in leaves, apical buds and flowers. For determining the function of *StFT* in tuberization, the expression of *StFT* in mRNA in tubering organs was detected. mRNA accumulation of *StFT* was obviously increased at the stage of tuber initiation, compared with other stages. This result shows that *StFT* might be involved in the stolon-to-tuber transition of potato.

Expression of *StFT* gene in *StCOL*-antisense transgenic potato plants

To verify the relationship of *StFT* and *StCOL* in tuberization, we analyzed the expression of *StFT* in the tubering organs of *StCOL*-antisense transgenic potato plants. The level of *StFT* transcript in the transgenic plants was so low that it was not detected in all tissues tested, contrasted to in the wild control plants (Fig.6). This result indicates that *StFT* expression is regulated by *StCOL*.

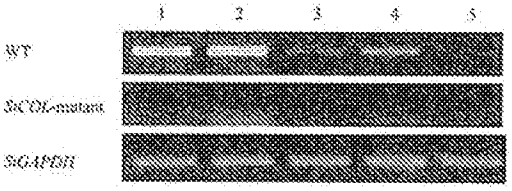


Fig. 6 Expression analysis of *StFT* in mutant of potato. 1, leaves; 2, apical buds; 3, stems; 4, stolons; 5, mature tubers. WT, wild-type, *StCOL*-mutant, mutant of antisense-*StCOL* transgenic potato.

DISCUSSION

It has been known that *AtFT* gene cDNA contained an open reading frame of 528 bp coding a protein of 175 amino acids which possessed a conserved PBP domain and its DNA sequence contained four exons and three introns (Kardailsky *et al.* 1999; Kobayashi *et al.* 1999). Our analysis showed that *StFT* and its coding protein were very similar to *AtFT* and AtFT from *Arabidopsis* in gene and protein structure. Phylogenetic analysis placed the StFT and the AtFT into the same cluster. This result suggests that StFT is a FT homologue.

FT function has been studied in several plants, especially in cereals (Kojima *et al.* 2002; Yan *et al.* 2006), tomato (Lifschitz *et al.* 2006), cucurbits (Lin *et al.* 2007), *Arabidopsis* (Notaguchi *et al.* 2008), and orchid (Hou and Yang 2009), which mainly concerns flower development. In our study, *StFT* transcript was detected in potato leaves, apical buds, flowers, stolons, and tubers. This result reveals that FT might also be involved in other biological processes of plant growth and development, in addition to regulating plant flowering.

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Photoperiod is an important environmental factor influencing tuberization in potato. CO is a transcriptional factor situated in the downstream of PHYB and plays a negatively regulating role in potato tuber formation (Rodriguez-Falcón *et al.* 2006). To verify the role of endogene CO in tuberization, we cloned *StCOL* gene (Guo *et al.* 2007) and constructed *StCOL*-antisense transgenic potato plants in previous experiment. The *StCOL* inhibitory expression in the *StCOL*-antisense plants caused normal tuberization under long-day conditions and an increase in tuber number under short-day conditions (data not shown). As one of the targets of CO, FT functions as an integrator of the different flowering regulatory pathways. In order to know whether it also plays a role on tuberization induction, in this study *StFT* expression in the *StCOL*-antisense plants was analysed. *StFT* mRNA was not detected in the stolons and tubers of these plants, while in its wild plants there was expression with variable levels. This result reveals that StFT is in the downstream of StCOL and might be involved in the *StCOL*-mediated regulation of potato tuberization.

CONCLUSION

In this paper, we reported a *StFT* gene cloned from potato cultivar Désirée. The gene was expressed in leaves, apical buds, flowers, and swelling stolons of the donor plants, but its transcript was not detected in all organs examined of *StCOL*-antisense transgenic potato plants, suggesting that *StFT* could be involved in the regulation of potato tuberization as downstream of *StCOL* gene, besides flowering induction.

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Rpi-vnt1.1, a *Tm-2*^{2} Homolog from *Solanum venturii*, Confers Resistance to Potato Late Blight

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Despite the efforts of breeders and the extensive use of fungicide control measures, late blight still remains a major threat to potato cultivation worldwide. The introduction of genetic resistance into cultivated potato is considered a valuable method to achieve durable resistance to late blight. Here, we report the identification and cloning of *Rpi-vnt1.1*, a previously uncharacterized late-blight resistance gene from *Solanum venturii*. The gene was identified by a classical genetic and physical mapping approach and encodes a coiled-coil nucleotide-binding leucine-rich repeat protein with high similarity to *Tm-2*^{2} from *S. lycopersicum* which confers resistance against *Tomato mosaic virus*. Transgenic potato and tomato plants carrying *Rpi-vnt1.1* were shown to be resistant to *Phytophthora infestans*. Of 11 *P. infestans* isolates tested, only isolate EC1 from Ecuador was able to overcome *Rpi-vnt1.1* and cause disease on the inoculated plants. Alleles of *Rpi-vnt1.1* (*Rpi-vnt1.2* and *Rpi-vnt1.3*) that differed by only a few nucleotides were found in other late-blight-resistant accessions of *S. venturii*. The late blight resistance gene *Rpi-phu1* from *S. phureja* is shown here to be identical to *Rpi-vnt1.1*, suggesting either that this strong resistance gene has been maintained since a common ancestor, due to selection pressure for blight resistance, or that genetic exchange between *S. venturii* and *S. phureja* has occurred at some time.

Potato (*Solanum tuberosum* L.) is the fourth most important crop and the most important noncereal food crop in the world (Lang 2001). The major biotic factor which limits potato crop yields is late blight, caused by the oomycete pathogen *Phytophthora infestans* (Mont.) de Bary. Famous as the cause of the Great Irish Potato Famine of the mid-nineteenth century (Large 1940), this devastating disease can result in complete loss of crop yield unless effective control measures are applied (Świeżyński and Zimnoch-Guzowska 2001). Fungicide treatment is currently the most common method for controlling late blight. However, sprays can be required every 4 days during severe epidemics and the high cost of fungicide application is a major burden to growers, especially in developing countries. Moreover, because fungicide application can have an impact on health and the environment, the use of many chemicals is becoming restricted. In addition, the pathogen evolves quickly and insensitivity or tolerance to commonly used fungicides can arise (Day and Shattock 1997; Goodwin et al. 1996). Therefore, the introduction of genetic resistance into cultivated potato is considered to be a valuable method to achieve durable resistance to late blight.

Two main types of resistance to late blight have been described in potato: field resistance and resistance (*R*) gene–mediated resistance (Umaerus and Umaerus 1994). Field resistance (also referred to as general or quantitative resistance) is frequently attributed to major quantitative trait loci (QTL) and a few minor QTL and generally results in partial resistance. Field resistance is considered by some to be more durable than resistance conferred by single *R* genes (Turkensteen 1993). However, partial resistance is also strongly correlated with maturity type and, thus, makes resistance breeding more difficult (Wastie 1991). Also, the genetic positions of QTL often correspond to regions of *R* gene clusters (Gebhardt and Valkonen 2001; Grube et al. 2000), raising the possibility that field resistance is due to the action of multiple weak *R* genes which are also susceptible to defeat by the evolving pathogen. Specific resistance is based on major dominant *R* genes. In early breeding programs during the first half of the twentieth century, 11 *R* genes (*R1* to *R11*) were identified in *S. demissum*, a wild species originating from Mexico. The *S. demissum* genes *R1, R3*, and *R10* have been heavily relied on for blight resistance in major breeding programs within Europe since their introgression. As a result, the *R* genes introgressed from *S. demissum* to cultivated potato lines have been overcome as new pathogen strains evolve that are virulent on the previously resistant hosts (Fry and Goodwin 1997; Umaerus and Umaerus 1994; Wastie 1991). This ability of *P. infestans* to rapidly overcome *R* genes limits the durability of any single *R* gene. Although some of the *S. demissum* genes such as *R5, R8*, and *R9* have not been used in European cultivars, isolates of *P. infestans* which overcome these genes are known, albeit rare. However, it is possible that, by deploying multiple *R* genes as a mixture in an otherwise genetically uniform crop, the ability of *P. infestans* to overcome these genes may be impaired (Jones 2001; Pink and Puddephat 1999).

S. J. Foster and T.-H. Park are joint first authors who contributed equally to the research.

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Nucleotide sequence data for the *Solanum venturii* late blight resistance genes *Rpi-vnt1.1, Rpi-vnt1.2*, and *Rpi-vnt1.3* are available in the GenBank database under accession numbers FJ423044, FJ423045, and FJ423046, respectively.

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Recent efforts to identify late-blight resistance have focused on major *R* genes conferring broad-spectrum resistance derived from diverse wild *Solanum* spp. In addition to *S. demissum*, other wild *Solanum* spp. such as *S. acaule*, *S. chacoense*, *S. berthaultii*, *S. brevidens*, *S. bulbocastanum*, *S. microdontum*, *S. sparsipilum*, *S. spegazzinii*, *S., stoloniferum*, *S. sucrense*, *S. toralapanum*, *S. vernei*, and *S. verrucosum* have been reported as new sources for resistance to late blight (Hawkes 1990; Jansky 2000).

Much progress has been made in the identification, mapping, and cloning of *R* genes against late blight. Of the *S. demissum R* genes, *R3*, *R6*, and *R7* have been mapped to a complex locus on chromosome XI (El-Kharbotly et al. 1994, 1996; Huang et al. 2004). The *R3* locus transpired to contain two distinct, closely linked genes, *R3a* and *R3b* (Huang et al. 2004, 2005). *R5, R8, R9, R10*, and *R11* appear to be allelic versions of the *R3* locus (Huang 2005) although *R10* is reported to behave more like a QTL than a dominant *R* gene (Bradshaw et al. 2006). *R1* has been mapped to chromosome V (El-Kharbotly et al. 1994; Leonards-Schippers et al. 1992) and *R2* to chromosome IV (Li et al. 1998). Of these, *R1* and *R3a* have been cloned (Ballvora et al. 2002; Huang et al. 2005). Of the resistances identified in wild *Solanum* spp., three *R* genes, *RB/Rpi-blb1*, *Rpi-blb2*, and *Rpi-blb3* from *S. bulbocastanum*, have been mapped to chromosomes VIII, VI, and IV, respectively (Naess et al. 2000; Park et al. 2005a; van der Vossen et al. 2003, 2005). *Rpi-abpt*, probably from *S. bulbocastanum*, has been localized on chromosome IV (Park et al. 2005c). *Rpi1* from *S. pinnatisectum* on chromosome VII (Kuhl et al. 2001), *Rpi-mcq1* (formerly *Rpi-moc1*) from *S. mochiquense* (Smilde et al. 2005), and *Rpi-phu1* from *S. phureja* on chromosome IX (Śliwka et al. 2006) have also been identified. Thus far, *Rpi-blb1* (*RB*) and *Rpi-blb2* are the only late-blight *R* genes reported to have been successfully cloned from wild *Solanum* spp. (Song et al. 2003; van der Vossen et al. 2003, 2005).

Here, we report the identification and cloning of the chromosome IX *Rpi* gene *Rpi-vnt1.1*, a previously uncharacterized late-blight resistance gene from *S. venturii*, an Argentinean wild species with an endosperm balance number (EBN) of 2. The gene was identified by a classical genetic and physical mapping approach and encodes a coiled-coil nucleotide-binding leucine-rich repeat (CC-NB-LRR) protein with high similarity to *Tm-2*^{2} from *S. lycopersicum* (formerly *Lycopersicon esculentum*) which confers resistance against *Tomato mosaic virus* (ToMV).

RESULTS

Variation for resistance to *P. infestans* in Centre for Genetics Resources accessions.

Accessions of *S. venturii* and *S. okadae* were obtained from the Centre for Genetics Resources in Wageningen, the Netherlands (CGN) (Table 1). The *S. venturii* accessions were originally listed as *S. okadae* in the CGN database but have recently been reclassified based on work using amplified fragment length polymorphism (AFLP) markers to study the validity of species labels in *Solanum* section Petota (Jacobs 2008; Jacobs et al. 2008). Species classification of accession CGN18279 is at present unclear because conflicting positions in the species dendrogram were obtained for this accession (Jacobs 2008). Screening of the accessions using *P. infestans* isolates 98.170.3 and 90128 in detached leaf assays showed phenotypic variation for resistance in four of the *S. venturii* accessions, *S. okadae* accession CGN18129, and the unclassified accession CGN18279 (Table 1). Resistance was evident as a complete lack of sporulation on leaf tissue whereas extensive mycelial growth was evident on leaves of sensitive individuals from 5 to 6 days postinoculation. Sensitive leaves often turned completely black by 7 days postinoculation. All individuals tested from the remaining accessions were sensitive to isolates 90128 and 98.170.3, despite CGN data indicating that at least three of these accessions were moderately or very resistant to *P. infestans*.

Development of *S. venturii* mapping populations.

Resistant individuals from five of the *S. venturii* accessions were crossed with sensitive individuals from either the same or different accessions (Table 2). In each of the crosses, resistance to *P. infestans* segregated 1:1 in the resulting progeny, indicating the presence of potentially five *Rpi* genes in the resistant heterozygous parents (Table 2).

Mapping *Rpi* genes in *S. venturii*.

Bulked segregant analysis (BSA) (Michelmore et al. 1991) was carried out on DNA pools comprising DNA from each of 10 resistant and susceptible progeny from the populations Svnt014 (*Rpi-vnt1.1*), Svnt013 (*Rpi-vnt1.2*), and Svnt040 (*Rpi-vnt1.3*) to find AFLP markers linked to these resistance loci. In total, 72 primer combinations were tested for each population and potentially linked markers were confirmed by checking for cosegregation of the marker with the resistance gene in the individual progeny that made up the bulks. A number of linked

Table 1. Reaction to *Phytophthora infestans* of *Solanum okadae* and *Solanum venturii* accessions used in this study

		Reference data		Fine screening[a]			
Accession[b]	Species[c]	Phenotype[d]	Source	R	MR	MS	S
CGN17998	*S. venturii*	Very resistant	CGN	2	...	...	7
CGN17999	*S. venturii*	Resistant	CGN	3	...	...	7
CGN18108	*S. venturii*	Very resistant	CGN	8	...	3	...
CGN18109	*S. venturii*	Very resistant	CGN	...	...	...	10
CGN22703	*S. venturii*	Very sensitive	CGN	4	1	...	4
CGN18269	*S. venturii*	Sensitive	CGN	...	...	...	10
CGN18279	Unclassified	Very resistant	CGN	4	...	...	5
CGN18129	*S. okadae*	Sensitive	CGN	...	2	2	6
CGN20599	*S. okadae*	Sensitive	CGN	...	...	...	10
CGN18157	*S. okadae*	Moderately resistant	CGN	...	...	...	10
CGN22709	*S. okadae*	Very sensitive	CGN	...	...	...	8
BGRC27158	*S. okadae*[e]	Moderately resistant	CGN	...	...	...	1

[a] Number of plants showing resistant (R), moderately resistant (MR), moderately sensitive (MS), or sensitive (S) phenotypes.
[b] CGN = Centre for Genetic Resources in the Netherlands; BGRC, Braunschweig Genetic Resources Center.
[c] Species designation according to Jacobs (2008).
[d] Blight resistance phenotype according to CGN data.
[e] Was not included in the study of Jacobs (2008); hence, original classification given.

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AFLP markers were placed on the *Rpi-vnt1.1, Rpi-vnt1.2*, and *Rpi-vnt1.3* linkage maps (Fig. 1), including SvntM2.9L (*Rpi-vnt1.1*); Svnt13M5.17, P12M44_103, P13M42_228, and P17M33_472 (*Rpi-vnt1.2*); and M6.44 (*Rpi-vnt1.3*). Of these, SvntM2.9L (6 centimorgans [cM] from *Rpi-vnt1.1*), Svnt13M5.17 (6.5 cM from *Rpi-vnt1.2*), and M6.44 (23 cM from *Rpi-vnt1.3*) were successfully converted into polymerase chain reaction (PCR)-based markers.

The *Rpi-vnt1.1* marker SvntM2.9L was also found to be polymorphic (by *Alu*I digestion) between *S. lycopersicum* (formerly *L. esculentum*) and *S. pennellii* (formerly *L. pennellii*), the parents of the *L. pennellii* introgression lines (Eshed and Zamir 1994). Screening of the individual introgression lines (IL) showed that this marker was located in IL 9.2, indicating that the marker could be on either arm of chromosome IX. The polymorphism was not present in IL 9.1, which overlaps IL 9.2 substantially, suggesting that the marker was situated proximal to the centromere on either chromosome arm. Further evidence for the location of the *Rpi-vnt* locus on chromosome IX came from the finding that polymorphisms in three chromosome IX simple-sequence repeat (SSR) markers (Stm0010, Stm 1051, and Stm 3012) (Milbourne et al. 1998) were linked to *Rpi-vnt1.3* (Fig. 1).

Additional chromosome IX markers were developed by designing PCR primers from known chromosome IX restriction fragment length polymorphism marker sequences within the SGN database, sequencing the PCR products amplified from both resistant and sensitive parental DNA and identifying single nucleotide polymorphisms (SNP) that could be used to develop cleaved amplified polymorphic sequence (CAPS) markers (Table 3). In this way, *Rpi-vnt1.1* was mapped to a 6.0-cM region of chromosome IX, delimited by markers C2_At4g02680 and TG186. *Rpi-vnt1.2* and *Rpi-vnt1.3* also mapped to the same location as *Rpi-vnt1.1* (Fig. 1). Although the markers TG551, TG35, T1421, C2_At3g63190, and C2_At4g02680 are bridging markers between the maps of at least two of the *Rpi* genes, the SNP present between the parents of the respective populations were not identical (as illustrated by the restriction enzymes used to reveal the polymorphisms) (Table 3), suggesting that each of the genes resides on a distinct haplotype. The markers TG551, TG35, and T1421 were also found to be polymorphic in the *Rpi-vnt1.4* and *Rpi-vnt1.5* populations. How-

Table 2. Crosses within *Solanum venturii* and late-blight-resistant (R) and -sensitive (S) segregants in their progenies

	Population parents[a]		Segregating progeny[b]		
Population	**Female**	**Male**	**R**	**S**	***Rpi* gene identified**
Svnt014	A618, CGN18108, R	A613, CGN18108, S	26	24	*Rpi-vnt1.1*
Svnt012	A622, CGN18279, S	A618, CGN18108, R	18	23	*Rpi-vnt1.1*
Svnt013	A624, CGN18279, R	A613, CGN18108, S	18	25	*Rpi-vnt1.2*
Svnt040	A606, CGN17998, R	A628, CGN18279, S	25	23	*Rpi-vnt1.3*
Svnt241	D986, BGRC08237, R	B419, Svnt012, S	24	26	*Rpi-vnt1.4*
Svnt184	D403, CGN17999, R	D401, CGN17999, S	24	21	*Rpi-vnt1.5*

[a] Plant identifier number followed by its accession number and reaction to *Phytophthora infestans* inoculation. CGN, Centre for Genetic Resources in the Netherlands; BGRC, Braunschweig Genetic Resources Center.
[b] Number of plants showing resistant (R) or sensitive (S) phenotypes.

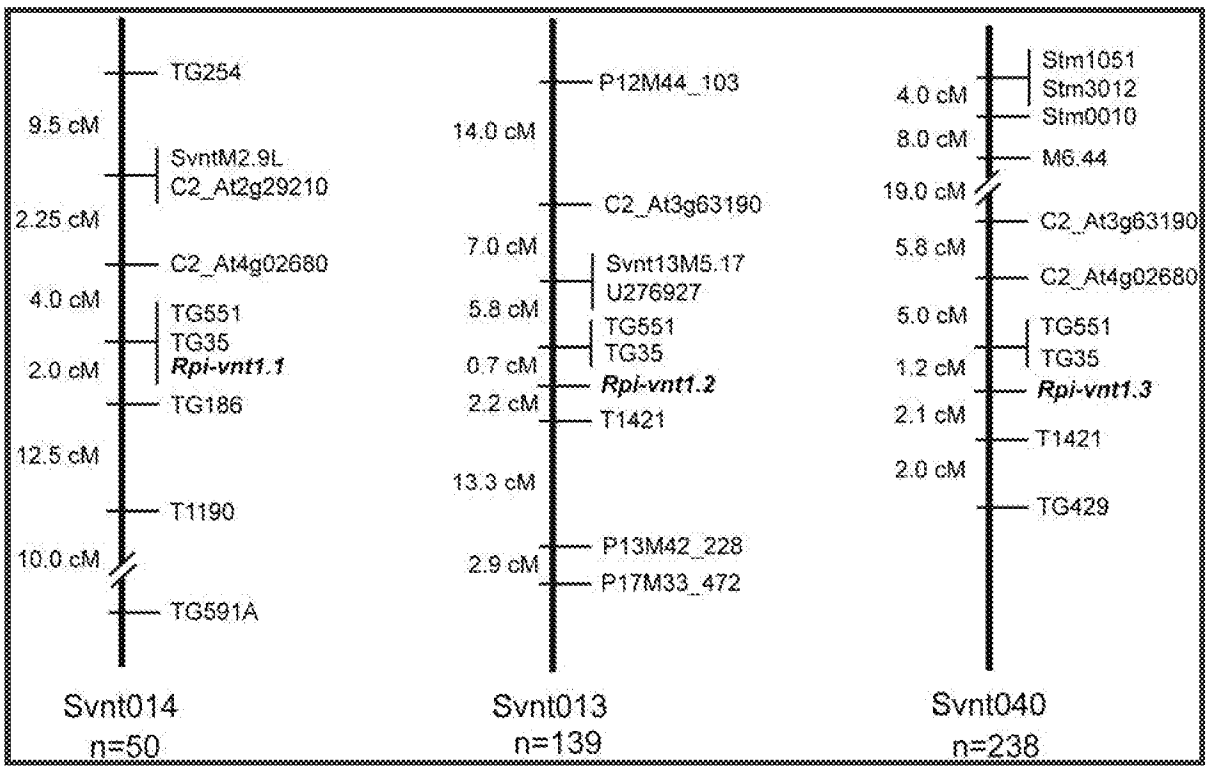


Fig. 1. Genetic linkage maps for *Rpi-vnt1.1, Rpi-vnt1.2*, and *Rpi-vnt1.3* on chromosome IX of *Solanum venturii*. Marker names and genetic distance in centimorgans (cM) are indicated on the right and left of each map, respectively. For each map, the number of individuals on which the mapping is based is given by *n*.

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ever, the SNP present in the markers were identical to those of the *Rpi-vnt1.1* haplotype and, thus, we assumed that the haplotype present in *S. venturii* accessions BGRC08237 and CGN17999 was identical to that of CGN18108, from which *Rpi-vnt1.1* is derived.

Concurrent work by Pel and associates (2009) used nucleotide-binding site (NBS) profiling (van der Linden et al. 2004) to identify a candidate NBS marker (NBS3a) with homology to the ToMV *R* gene *Tm-2*^{2} (Lanfermeijer et al. 2003) which was closely linked to *Rpi-vnt1.1* in a population derived from the same *S. venturii* accession (CGN18108). From the sequence of this marker, we designed allele-specific PCR primers (vntNBSHae-F/R) (Table 3) and showed that this marker (vntNBSHae) cosegregated with *Rpi-vnt1.1* and *Rpi-vnt1.2* and mapped 1.2 cM distal to *Rpi-vnt1.3* (Fig. 1).

BAC library screening and contig construction.

The *S. venturii* plant K39 which was used to construct the BAC (bacterial artificial chromosome) library is a transheterozygote carrying both *Rpi-vnt1.1*, originally from the resistant parent plant A618 (accession CGN18108), and *Rpi-vnt1.2* from the resistant plant A624 (accession CGN18279). The *S. venturii* K39 BAC library consisted of 105,216 clones stored in 274-by-384-well microtiter plates. Insert size ranged from

Table 3. Polymerase chain reaction-based markers used for mapping of *Rpi-vnt1.1*, *Rpi-vnt1.2*, and *Rpi-vnt1.3*

			Type of marker[a]		
Marker	**Primer sequence (5′–3′)**	**Temperature (°C)**	***Rpi-vnt1.1***	***Rpi-vnt1.2***	***Rpi-vnt1.3***
vntNBSHae	F: CTTACTTTCCCTTCCTCATCCTCAC R: TGAAGTCATCTTCCAGACCGATG	60	a.s.	a.s.	*Mae*III
vnt1Long	F: AGTTATACACCCTACATTCTACTCG R: CTTTGAAAAGAGGCTTCATACTCCC	60	a.s.	a.s.	a.s.
vnt1ORF	F: GGG**CTCGAG**CGAAATACCAGCTAACAAAAGATG[b] R: GGC**GGATCC**TAGTACCTGTGATATTCTCAACTTTGC[c]	60	a.s.	a.s.	a.s.
TG254	F: AGTGCACCAAGGGTGTGAC R: AAGTGCATGCCTGTAATGGC	60	…	…	…
At2g38025	F: ATGGGCGCTGCATGTTTCGTG R: ACACCTTTGTTGAAAGCCATCCC	55	…	…	*Tsp509*I (R)
Stm1051	F: TCCCCTTGGCATTTTCTTCTCC R: TTTAGGGTGGGGTGAGGTTGG	55	…	…	SSR
Stm3012	F: CAACTCAAACCAGAAGGCAAA R: GAGAAATGGGCACAAAAAACA	55	…	…	SSR
Stm0010	F: TCCTTATATGGAGCAAGCA R: CCAGTAGATAAGTCATCCCA	50	…	…	SSR (R)
M6.44	F: ATTGAAAGAATACACAAACATC R: ATTCATGTTCAGATCGTTTAC	55	…	…	*Dde*I
At3g63190	F: TTGGTGCAGCCGTATGACAAATCC R: TCCATCATTATTTGGCGTCATACC	55	…	*Eco*RI	*Tsp509*I
SvntM2.9L	F: ACAAACCTATGTTAGCCTCCCACAC R: GGCATCAAGCCAATGTCGTAAAG	60	*Dde*I	…	…
At2g29210	F: AGCAGGACACTCGATTCTCTAATAAGC R: TGCACTAAGTAGTAATGCCCAAAGCTC	55	*Nco*I	…	…
Svnt13M5.17	F: CTGAGGTGCAGCCAATAAC R: CCAGTGAGAAACAGCTTCTC	55	…	a.s.	…
U276927	F: GATGGGCAACGATGTTGTTG R: GCATTAGTACAGCGTCTTGGC	60	…	*Hpy188*I	…
At4g02680	F: GTGAAGAAGGTCTACAGAAAGCAG R: GGGCATTAATGTAGCAATCAGC	55	*Mse*I	…	*Nhe*I
TG551	F: CATATCCTGGAGGTGTTATGAATGC R: AACTTTCAGATATGCTCTGCAGTGG	60	*Mwo*I	*Taq*I	*Taq*I
TG35	F: CACGGAGACTAAGATTCAGG R: TAAAGGTGATGCTGATGGGG	55	*Hha*I	*Alu*I	*Tsp509*I
T1421	F: CATCAATTGATGCCTTTGGACC R: CTGCATCAGCTTCTTCCTCTGC	60	…	*Bsl*I	*Rsa*I
TG186	F: AATCGTGCAGTTTCAGCATAAGCG R: TGCTTCCAGTTCCGTGGGATTC	60	*Dra*I (R)	…	…
TG429	F: CATATGGTGACGCCTACAG R: GGAGACATTGTCACAAGG	55	…	…	*Mse*I
T1190	F: GTTCGCGTTCTCGTTACTGG R: GTTGCATGGTTGACATCAGG	55	a.s.	…	…
TG591A	F: CTGCAAATCTACTCGTGCAAG R: CTCGTGGATTGAGAAATCCC	60	a.s.	…	…
266I9F	F: GTATGTTTGAGTTAGTCTTCC R: TATAATAGGTGTTCTTGGGG	55	…	*Hin*fI	…
266I9R	F: AAGGTGTTGGGAGTTTTTAG R: TATCTTCCTCATTTTGGTGC	55	*Hin*dIII	*Hin*dIII	…
185L21R	F: GATTGAGACAATGCTAGTCC R: AGAAGCAGTCAATAGTGATTG	55	*Bsl*I	*Rsa*I	…
148P20R	F: AAGATTCTTTTTCCTCCTTAG R: AAAGATGAAGTAGAGTTTTGG	58	*Hpy*CH4IV	…	…

[a] Restriction enzymes indicate that marker is a cleaved amplified polymorphic sequence marker, a.s. indicates allele-specific markers, R indicates that marker is linked in repulsion phase, SSR indicates that marker is a simple-sequence repeat marker, and … indicates that the marker was either not polymorphic or not tested for that *Rpi* gene.

[b] Bold type indicates *Xho*I site included in 5′ region for cloning purposes.

[c] Bold type indicates *Bam*HI site included in 5′ region for cloning purposes.

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60 to 165 kb with an average of 103.5 kb, based on pulsed-field gel analysis of *Not*I-digested DNA from randomly selected clones. The haploid genome size of *S. venturii* is estimated to be approximately 1,000 Mb; therefore, the library was predicted to represent a coverage of approximately 11 genome equivalents.

PCR markers TG551, TG35, and vntNBSHae linked to *Rpi-vnt1.1* and *Rpi-vnt1.2* were used to screen the *S. venturii* K39 BAC library. BAC clones identified using these markers were end sequenced and a contig was constructed using a BAC-end PCR strategy (Fig. 2). Marker vntNBSHae is an allele-specific marker linked to both *Rpi-vnt1.1* and *Rpi-vnt1.2* and, consequently, it was not possible to assign haplotype to the BAC clones identified using this marker. However, using many of the BAC end markers and the CAPS markers TG551 and TG35, BAC clones from the *Rpi-vnt1.1* and *Rpi-vnt1.2* haplotypes can be distinguished by restriction digestion (Table 3). Two of the clones from the K39 library (K39_272N11 with *Rpi-vnt1.2* haplotype and K39_256M23 with *Rpi-vnt1.1* haplotype) had BAC end sequences which were highly similar to each other and to the ToMV *R* gene *Tm-2*^{2} (Lanfermeijer et al. 2003) and were identical to the vntNBSHae marker.

As a parallel contig construction approach, BAC clones containing sequences homologous to the vntNBSHae marker were identified by hybridization of the vntNBSHae probe to the pooled BAC DNA (274 pools of 384 clones each). In all, 67 pools were identified as containing BAC clones with homologous sequences. Clones from the positive pools were double-spotted at high density onto nylon membranes and hybridized with the vntNBSHae probe to identify individual BAC clones from the pools. In total, 85 BAC clones were identified. DNA from identified BACs was subjected to BAC SNaPshot fingerprinting (Luo et al. 2003), along with an additional 10 selected clones which were positive for the TG551 or TG35 markers. Analysis of the SNaPshot fingerprinting patterns using FPC v4.7 (Soderlund et al. 2000) showed that the BAC clones formed nine distinct contigs containing between 1 to 22 clones. From the contigs generated, one contig contained the BAC clones identified by both PCR-based screening using the linked markers TG551, TG35, and vntNBSHae and hybridization using the vntNBSHae marker as a probe (Fig. 2).

High-resolution mapping and cloning of *Rpi-vnt1.1, Rpi-vnt1.2*, and *Rpi-vnt1.3.*

PCR primers were designed from BAC end sequences and used to amplify products from the parental genotypes of *Rpi-vnt1.1* and *Rpi-vnt1.2.* PCR products were sequenced and analyzed for the presence of SNP that allowed use of the PCR products as CAPS markers in the respective populations. In all, 60 recombinants between the closest flanking markers SvntM2.9L and TG186 were selected from the total *Rpi-vnt1.1* population of 1,213 individuals and successfully converted BAC end markers were used to construct a higher-resolution genetic map for *Rpi-vnt1.1.* Similarly, 46 recombinants (between TG551 and T1421) from the *Rpi-vnt1.2* population of 1,706 individuals were used to construct a higher-resolution *Rpi-vnt1.2* genetic map. The positions of these markers in relation to marker vntNBSHae, which co-segregates with both *Rpi-vnt1.1* and *Rpi-vnt1.2*, are shown in Figure 2.

Marker analysis indicated that *Rpi-vnt1.1* was located within a genetic interval of 0.33 cM delimited by the CAPS marker TG35 and the BAC end marker 185L21R (Fig. 2). By reference to the physical map constructed from PCR and SNaPshot fingerprinting analysis of BAC clones from the K39 library (Fig. 2), *Rpi-vnt1.1* was predicted to be present on a physical region covered by the BAC clones K39_148P20, K39_266I9, and K39_185L21 (Fig. 2). Low-stringency Southern blotting of these clones revealed that a single fragment homologous to the vntNBSHae marker was present in both K39_266I9 and K39_185L21, indicating that there was potentially a single CC-NB-LRR gene located in the overlap region between these two clones. BAC clones K39_266I9 and K39_148P20 were sequenced and one candidate open reading frame (ORF) was

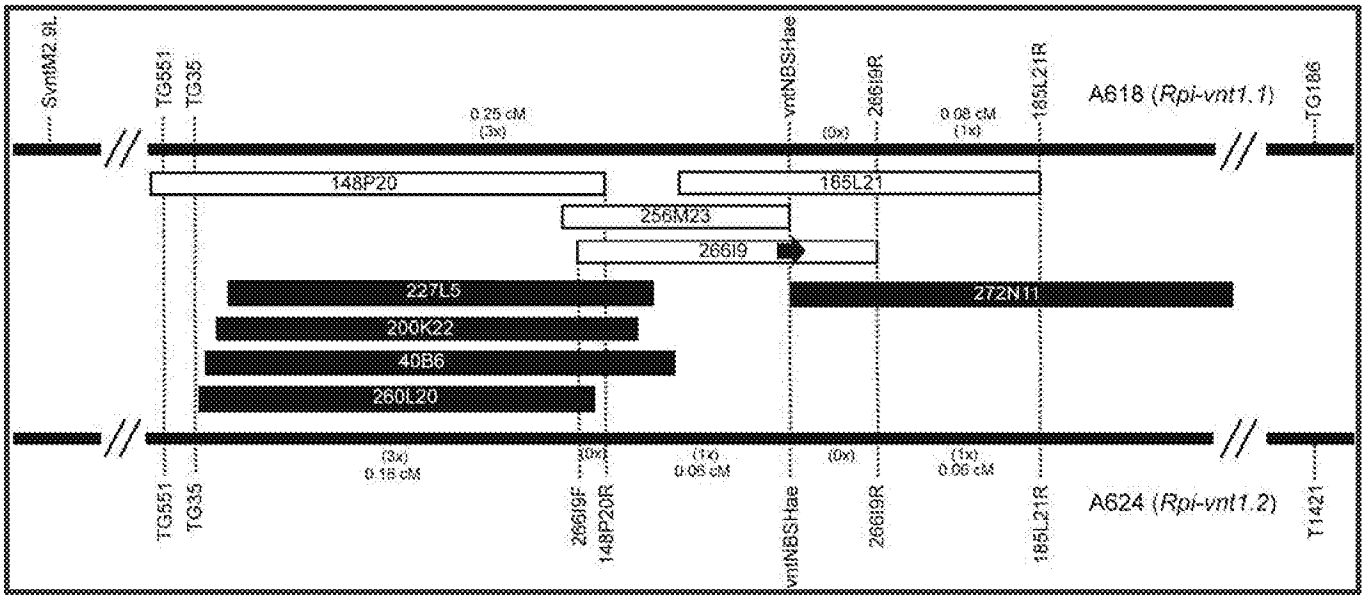


Fig. 2. High-resolution genetic mapping and physical mapping of *Rpi-vnt1.1* and *Rpi-vnt1.2* using the K39 BAC (bacterial artificial chromosome) library. Diagram shows the alignment of BAC clones from both the *Rpi-vnt1.1* and *Rpi-vnt1.2* haplotypes around the marker vntNBSHae, which cosegregates with resistance. BAC clone 148P20 was identified using markers TG551 and TG35; clones 185L21, 256M23, 266I9, and 272N11 were identified using marker vntNBSHae as an allele-specific polymerase chain reaction marker and also as a hybridization probe; clones 227L5, 200K22, and 40B6 were identified using the BAC end markers 266I9F and 148P20R; and clone 260L20 was identified using the BAC end marker 266I9F. Open bars represent BAC clones from the *Rpi-vnt1.1* haplotype and black bars represent BAC clones from the *Rpi-vnt1.2* haplotype. The black arrow within BAC clone 266I9 illustrates the position of *Rpi-vnt1.1*. Vertical dotted lines indicate the positions of markers linked to resistance. Genetic distances are given in centimorgans (cM) and the numbers in parentheses represent the number of recombinants between markers. Markers SvntM2.9L, TG186, and T1421 are included to aid orientation of the physical map with the genetic map in Figure 1.

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identified for *Rpi-vnt1.1* on K39_266I9. No candidate *R* genes were present on BAC clone K39_148P20.

Interestingly, two individuals from the preselected *Rpi-vnt1.1* recombinants were resistant despite genotyping results suggesting that they should be susceptible. The mapping data for these two genotypes suggests that there may be a second, closely linked *R* gene located proximal to marker TG35.

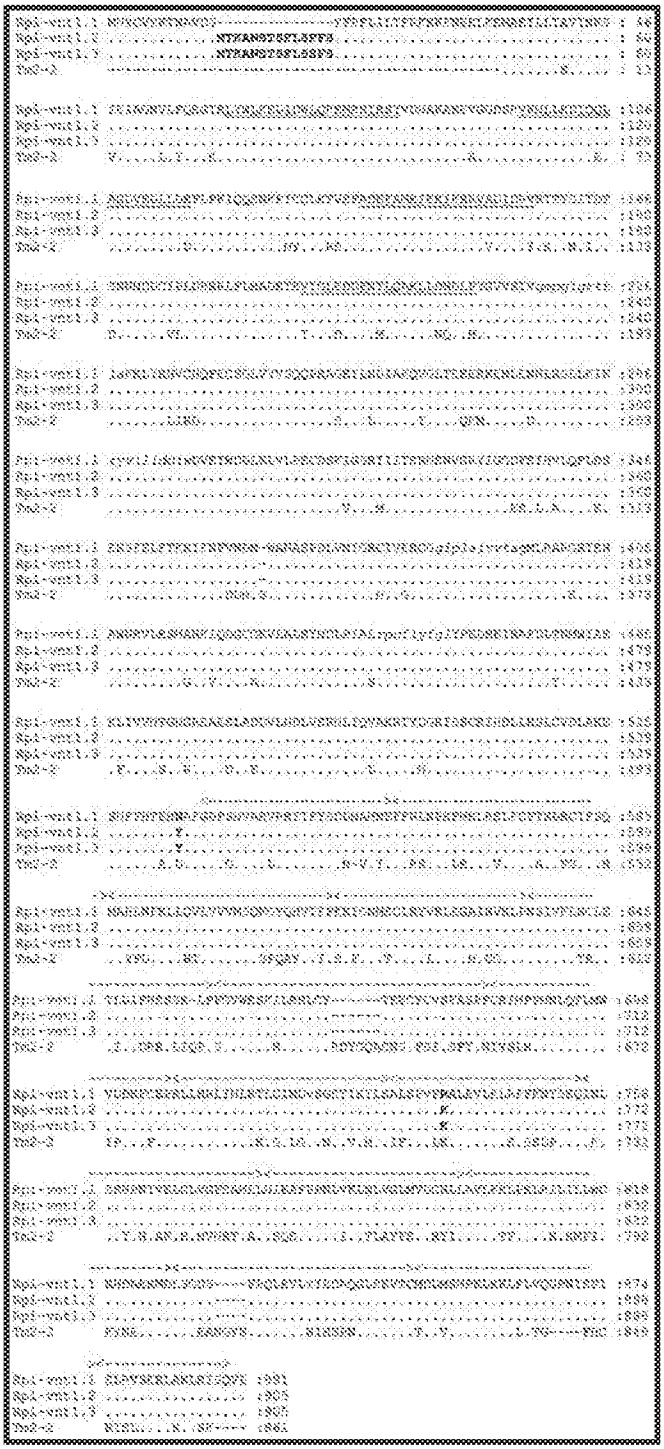


Fig. 3. Alignment of the deduced protein sequences of *Rpi-vnt1.1, Rpi-vnt1.2, Rpi-vnt1.3*, and *Tm-2*^{2} (AAQ10736). The complete amino acid sequence of Rpi-vnt1.1 is shown and dots indicate identical residues in the other two proteins. Where residues from Rpi-vnt1.2 and Tm-2^2 differ from Rpi-vnt1.1, the residues in these proteins are given. Amino acid differences between Rpi-vnt1.1, Rpi-vnt1.2, and Rpi-vnt1.3 are indicated in bold type. Predicted coiled-coil domains are underlined and the first and fourth hydrophobic residues of each heptad repeat are double underlined. Conserved motifs within the nucleotide-binding apoptosis, *R* gene products, CED-4 domain are indicated in lowercase italics. Putative leucine-rich repeats are indicated above the sequence line.

High-resolution mapping indicated that *Rpi-vnt1.2* was located within a genetic interval of 0.12 cM delimited by the BAC end markers 266I9F and 185L21R (Fig. 2). By reference to the physical map constructed from PCR and fingerprinting analysis of BAC clones from the K39 library, *Rpi-vnt1.2* was predicted to be present on the same physical region as that identified for *Rpi-vnt1.1*. However, other than the BAC clone K39_272N11, one end of which contained a partial *Tm-2*^{2} homolog which was identical to the corresponding region in *Rpi-vnt1.1*, clones from the *Rpi-vnt1.2* haplotype which covered the same physical region as *Rpi-vnt1.1* could not be identified within the library (Fig. 2). As an alternative approach, primers designed to amplify the complete *Rpi-vnt1.1* ORF (vnt1ORF-F/R) (Table 3) were used to genotype the five recombinant individuals between markers TG551 and T1421 from the *Rpi-vnt1.2* population. The resulting PCR product cosegregated with resistance in the three resistant individuals from these recombinants. The PCR product amplified from plant A624, the resistant parent plant of the *Rpi-vnt1.2* population, was cloned into pGEM-T Easy (Promega Corp., Madison, WI. U.S.A.) and was sequenced.

Rpi-vnt1.3 also mapped to the same genetic location as *Rpi-vnt1.1* and *Rpi-vnt1.2*; therefore, rather than initiate high-resolution mapping, the PCR primers designed against *Rpi-vnt1.1* were employed to amplify *Rpi-vnt1.3* candidates. The resulting PCR products were cloned into pGEM-T Easy (Promega Corp.) and sequenced. To confirm that the sequenced PCR product corresponded to *Rpi-vnt1.3,* seven recombinants between markers TG35 and T1421 (Fig. 1) were assessed by PCR. The candidate PCR product was only amplified from the resistant recombinants.

Analysis of *Rpi-vnt1.1, Rpi-vnt1.2*, and *Rpi-vnt1.3*.

The *Rpi-vnt1.1* ORF is 2,673 bp long and translates into a protein sequence of 891 amino acids with a calculated molecular weight of 102 kDa and a pI of 8.05. The *Rpi-vnt1.2* ORF comprises 2,715 bp and translates into a protein sequence of 905 amino acids with a calculated molecular weight of 103.6 kDa and a pI of 8.16. The sequence of *Rpi-vnt1.2* differs from *Rpi-vnt1.1* by an insertion of 42 nucleotides in the 5′ end of the gene (Fig. 3). The resulting additional 14 amino acids present in the corresponding region of *Rpi-vnt1.2* do not affect any of the predicted CC domains. There are also three SNP between *Rpi-vnt1.1* and *Rpi-vnt1.2*; A1501T, T1767C, and G2117A (Fig. 3). These nucleotide differences result in two amino acid differences between *Rpi-vnt1.1* and *Rpi-vnt1.2* (Fig. 3). The difference at position 501 is at the end of the NB-ARC (apoptosis, *R* gene products, CED-4) domain, just prior to the LRR region, and results in the change of an asparagine in *Rpi-vnt1.1* to a tyrosine in *Rpi-vnt1.2*. This amino acid change does not affect any of the characterized NB-ARC domains. At position 706, within the ninth LRR, an arginine in *Rpi-vnt1.1* becomes a lysine in *Rpi-vnt1.2*; both of these residues are positively charged polar amino acids and, hence, this can be considered a synonymous change.

The Rpi-vnt1.1 proteins contain all the features characteristic of the CC-NB-LRR class of resistance proteins. Within the first 215 (229 for Rpi-vnt1.2) amino acids of the N-terminal part of the protein were four regions each with three predicted heptad repeat motifs typical of CC domains (Fig. 3). All NB-ARC domains (van der Biezen and Jones 1998) are present in the amino acid sequence from 216 to 505 (230 to 519 in Rpi-vnt1.2). Following the NB-ARC domain is a region consisting of a series of 15 irregular LRR motifs that could be aligned according to the consensus sequence LxxLxxLxxLxLxxC/N/

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Sx (x)LxxLPxx (where L can be L, I, M, V, Y, or F and x is any amino acid) (McHale et al. 2006).

Rpi-vnt1.1 and *Rpi-vnt1.2* share 80.9 and 79.7%, identity, respectively, with *Tm-2*^{2} at the nucleic acid level. At the amino acid level, this translates to 72.1 and 71.1% identity, respectively. As expected, given its role in recognition specificity, the percentage of similarity was lowest in the LRR domain where *Rpi-vnt1.1/1.2* and *Tm-2*^{2} share only 57.5% similarity. In contrast, the sequence similarity across the CC and NB-ARC domains of *Rpi-vnt1.1/1.2* and *Tm-2*^{2} is 81.8 and 79.7%, respectively; notably, within the conserved domains of the NB-ARC region, *Tm-2*^{2} and *Rpi-vnt1.1* are identical.

The primers vnt1long-F and vnt1long-R (Table 3) were used to amplify *Rpi-vnt1.1* homologous sequences from the parental material containing *Rpi-vnt1.3*. Resulting PCR products were cloned into pGEM-T and sequenced. The sequences obtained were identical to *Rpi-vnt1.2* with the exception of a single SNP in the 5′ insertion region, relative to *Rpi-vnt1.1*, which resulted in a phenylalanine to serine amino acid substitution (Fig. 3).

It was not possible to amplify full-length *Rpi-vnt1.1* paralogues from the susceptible *S. venturii* parent A613. This observation, together with the fact that the vntNBSHae marker could only be amplified from resistant genotypes, could be taken to suggest that, at least in the susceptible parent used in this study, the susceptible phenotype is caused by an absence of *Rpi-vnt1.1* rather than a nonfunctional copy. An alternative explanation could be that the insertion of a transposable element into the susceptible allele has rendered it recalcitrant to PCR amplification or that sequence at the sites against which primers were designed is sufficiently different so as to prevent successful amplification.

Complementation analysis.

Potato cv. Desiree was transformed with plasmid pSLJ21152 which contained *Rpi-vnt1.1* under the control of its native promoter and terminator. In total, 37 *S. tuberosum* cv. Desiree plants capable of growth on kanamycin were selected as putative *Rpi-vnt1.1* transformants. Following transfer to the glasshouse, leaves from 29 plants were excised and used in a detached leaf assay with *P. infestans* isolates 90128 and BPC2006 3928A (superblight, blue 13) to determine whether the transgene conferred blight resistance (Fig. 4). Of the 29 transformants tested, 24 were confirmed as being resistant and did not show any signs of blight infection. Some plants exhibited signs of a hypersensitive response localized to the inoculation site. The remaining five plants were susceptible to both isolates, as was the control (nontransformed Desiree). The phenotype of the transgenic plants correlated exactly with amplification of the *Rpi-vnt1.1* ORF by PCR; all plants from which *Rpi-vnt1.1* could be amplified were confirmed as resistant. Detached leaves of selected transgenic lines of potato cv. Desiree carrying *Rpi-vnt1.1* were inoculated with a range of *P. infestans* isolates to determine the range of isolates against which *Rpi-vnt1.1* confers resistance (Table 4). Of the 11 isolates tested, only isolate EC1 from Ecuador was able to overcome *Rpi-vnt1.1* and cause disease on the inoculated plants. The resistant parent from which *Rpi-vnt1.1* was isolated was also shown to be susceptible to isolate EC1, demonstrating that the specificity of *Rpi-vnt1.1* was retained in the transgenic plants and that the resistance phenotype was not due to constitutive activation of defense pathways by the transgene.

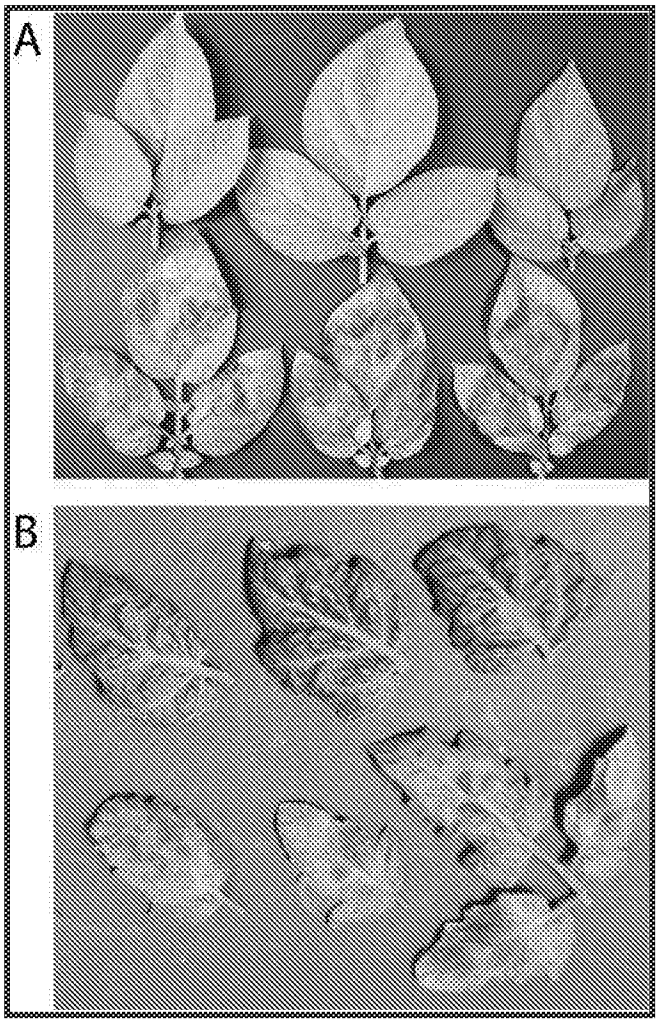


Fig. 4. *Rpi-vnt1.1* confers resistance to *Phytophthora infestans* upon transformation into susceptible potato and tomato cultivars. Detached leaves from plants of **A,** *Solanum tuberosum* cv. Desiree containing (top) or lacking (bottom) *Rpi-vnt1.1* and **B,** *S. lycopersicum* cv. Moneymaker containing (top) or lacking (bottom) *Rpi-vnt1.1* were inoculated at five to six points with 10-µl droplets of zoospore suspensions of *P. infestans* isolate 90128 and incubated at 16°C, 16 h of light, and 8 h of darkness. Photographs were taken 6 days postinoculation.

Table 4. Response of transgenic potato plants containing *Rpi-vnt1.1* against a range of *Phytophthora infestans* isolates

Isolate	Country of origin	Race	*Rpi-vnt1.1* phenotype
90128	The Netherlands	1.3.4.7.8.9.10.11	Resistant
IPO-0	Unknown	Unknown	Resistant
IPO-Complex	Belgium	1.2.3.4.6.7.10.11	Resistant
BPC2006 3928A (superblight, blue 13)	United Kingdom	Unknown	Resistant
Hica	United Kingdom	Unknown	Resistant
MP717	Poland	1.2.3.4.5.6.7.9.10.11	Resistant
MP778	Poland	1.3.4.5.6.7.9.10.11	Resistant
MP674	Poland	1.2.3.4.5.6.7.10.11	Resistant
MP622	Poland	1.3.4.7.8.10.11	Resistant
MP650	Poland	1.2.3.4.5.7.8.10.11	Resistant
EC1	Ecuador	2.4.10.11	Susceptible

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Transgenic tomato cv. Moneymaker plants carrying *Rpi-vnt1.1* were also shown to be resistant to *P. infestans* isolate 90128 (Fig. 4).

Rpi-vnt1.1 is also present in *S. venturii* accession CGN18000 and cosegregates with resistance in plants containing *Rpi-phu1.*

We identified an *Rpi* gene in a segregating population derived from a resistant individual of the *Solanum* accession CGN18000 which also showed close linkage between the identified gene (originally named *Rpi-nrs1*) and marker TG551 and cosegregated with marker vntNBSHae, indicating that it was located in the same region as *Rpi-vnt1.1* (data not shown). The same genetic location for this gene was also determined by Pel and associates (2009). Although listed in the CGN collection as *S. neorossii,* AFLP fingerprinting work showed that accession CGN18000 clustered with accessions of *S. venturii* and not *S. neorossii* (Jacobs 2008; Jacobs et al. 2008). Sequencing of a PCR product amplified using primers that amplify full-length *Rpi-vnt1.1* from resistant CGN18000 material showed that a sequence identical to *Rpi-vnt1.3* (i.e., with a single SNP in the 5′ region compared with *Rpi-vnt1.2*) was present in this material and cosegregated with resistance in the CGN1800 mapping population. Resistant CGN18000 material also showed the same response as *Rpi-vnt1.1* to the panel of *P. infestans* isolates. Interestingly, genotypes from the CGN18000 mapping population were identified which were found not to contain *Rpi-vnt1.3* by PCR yet were resistant in detached leaf assays, suggesting the presence of a second, linked gene reflecting the situation with *Rpi-vnt1.1.*

Rpi-phu1 from *S. phureja* was also reported to map to this region (Śliwka et al. 2006). The *Rpi-vnt1.1* marker vntNBSHae-F/R cosegregated with resistance in a population of 148 diploid *S. tuberosum* plants segregating for *Rpi-phu1.* Full-length *Rpi-vnt1.1* paralogues were amplified from DNA of three resistant genotypes containing *Rpi-phu1.* In each case, a single PCR product was obtained and sequencing showed this to be identical to *Rpi-vnt1.1.* Resistant plant material containing *Rpi-phu1* was also shown to be resistant to the *P. infestans* isolates used in this study, with the exception of EC1.

DISCUSSION

Despite the efforts of breeders and the extensive use of fungicide control measures, late blight still remains a major threat to potato cultivation worldwide. The spread of new isolates, including the A2 mating type, from Mexico to Europe from 1984 onward (Goodwin and Drenth 1997) has resulted in a European late blight population which is capable of sexual reproduction and, consequently, has the potential for strains of the pathogen to evolve and overcome *R* genes present within current cultivated potato germplasms which were introgressed from *S. demissum* in the 1930s (Black et al. 1953; Malcolmson and Black 1966). Consequently, there is a need to source new *R* genes and extend the repertoire available. South American wild *Solanum* spp. are a valuable source of *R* genes which have potentially novel recognition specificities and, thus, may prove to be valuable tools in the fight against late blight (Bradshaw and Ramsay 2005). Here, we have identified, mapped, and cloned such a gene from *S. venturii* and shown that this gene can be used to transfer resistance to the susceptible cv. Desiree.

Rpi-vnt1.1 was cloned using a genetic mapping and positional cloning approach. BSA (Michelmore et al. 1991) together with AFLP (Vos et al. 1995) were proven to be a powerful combination of tools for efficiently mapping *Rpi-vnt1.1* and *Rpi-vnt1.2.* In addition, the tomato introgression lines (Eshed and Zamir 1994) were invaluable in allowing the rapid location of the chromosomal positions of these genes, as was found previously for *Rpi-mcq1* (Smilde et al. 2005). *Rpi-vnt1.1* is located on the long arm of chromosome IX and shows highest similarity to *Tm-2*^{2} from *S. lycopersicum*, which confers resistance against ToMV and is located in a centromeric position on the long arm of chromosome IX (Lanfermeijer et al. 2003).

Both *Rpi-vnt1.1* and *Tm-2*^{2} belong to the largest family of *R* genes, which contain a characteristic CC domain in addition to the canonical NB-ARC and LRR domain. The majority of *R* genes cloned from potato are members of this CC-NB-LRR family and all *Rpi* genes cloned thus far are of this class. A member of the toll/interleukin 1 receptor (TIR)-NB-LRR family of *R* genes (characterized by the presence of a TIR domain) has been shown to be responsible for resistance against the nematode *Globodera rostochiensis* in potato (Paal et al. 2004); however, whether or not members of the TIR-NB-LRR family confer resistance against *P. infestans* remains to be seen.

Mapping results from two recombinants within the *Rpi-vnt1.1* locus indicated that a second closely linked *Rpi* gene may be located proximal to *Rpi-vnt1.1* in *S. venturii* accession CGN18108. This gene also confers resistance against *P. infestans* isolate 90128; however, whether this gene has exactly the same spectrum of recognition specificity as *Rpi-vnt1.1* remains to be seen. It was not possible to amplify either the marker vntNBSHae or the full-length *Rpi-vnt1.1* ORF from these genotypes, which suggests that the gene is somewhat different from *Rpi-vnt1.1* at the sequence level. Mapping using populations derived from these genotypes is required to investigate this gene further.

Although no other *R* genes were found within the 185 kb that comprised the BAC clones sequenced during the search for *Rpi-vnt1.1*, the presence of another closely linked *R* gene or cluster of *R* genes would not be unprecedented. All of the *Rpi* genes cloned to date are members of complex loci composed of *R* gene clusters. In the case of *Rpi-blb1*, *Rpi-blb2, R1*, and *R3a,* the other *R* genes present in the clusters did not necessarily confer late-blight resistance, although this possibility could not be ruled out (Ballvora et al. 2002; Huang et al. 2005; Song et al. 2003; van der Vossen et al. 2003, 2005). However, *R2, Rpi-blb3*, and *Rpi-abpt* (all of which confer resistance against a different spectrum of races of *P. infestans*) are found together in a single complex locus on chromosome IV (Park et al. 2005a).

Although no paralogues are found in the immediate vicinity of *Rpi-vnt1.1,* chromosome IX harbors a number of *Tm-2*^{2} paralogues; in addition to *Rpi-vnt1.1*, a contig of BAC clones generated by SNaPshot fingerprinting of BACs from the K39 BAC library also contains paralogous sequences and maps to a region on the distal part of chromosome IX, in the region of marker TG591B (which is itself a *Tm-2*^{2} homolog). This contig contains an estimated three or four *Rpi-vnt1.1* paralogues according to Southern blotting results (data not shown). *Rpi-mcq1,* which has recently been shown to be a *Tm-2*^{2} homolog with high similarity to *Rpi-vnt1.1*, also maps to this locus (Z. Chu, *unpublished data*). Thus, it appears that there is a family of *Rpi-vnt1.1*/ *Tm-2*^{2} paralogues distributed along the length of the long arm of chromosome IX. Additionally, screening of the *S. venturii* BAC library revealed the presence of at least six other contigs containing paralogues. The location of these on the genetic map is currently unknown.

A number of other resistance genes against pests and pathogens of potato and tomato are also found on chromosome IX. *Gm,* a gene which confers resistance against *Potato virus M* (Marczewski et al. 2006), has recently been mapped to a region similar to that occupied by *Rpi-vnt1.1* but it is presently not sufficiently mapped to determine whether it occupies the same locus in *S. gourlayi*, the donor of *Gm.* Proximal to *Rpi-vnt1.1* and the aforementioned *Tm-2*^{2} are *Fr1*, which confers

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resistance against *Fusarium oxysporum* f. sp. *radicis-lycopersici* in tomato (Vakalounakis et al. 1997), and *Ve1*, which confers resistance against *Verticillium dahliae* in both potato and tomato (Kawchuk et al. 2001). Distal to *Rpi-vnt1.1* are *Sw-5*, which confers resistance against *Tomato spotted wilt virus* (Brommonschenkel and Tanksley 1997; Brommonschenkel et al. 2000); *Nx*, conferring resistance against *Potato virus X* (Tommiska et al. 1998) in *S. phureja*; and a QTL for resistance against the nematode *G. pallida* in potato, designated *Gpa6* (Rouppe van der Voort et al. 2000). *Rpi-mcq1* (formerly *Rpi-moc1*) (Smilde et al. 2005) lies distal to *Rpi-vnt1.1* at the end of the short arm of chromosome IX, in a region similar to that to which *Ny-1,* conferring resistance to *Potato virus Y*, has also been mapped (Szajko et al. 2008).

The late blight resistance gene *Rpi-phu1* (Śliwka et al. 2006) was mapped within a segregating diploid mapping population derived from complex *Solanum* hybrids of *S. tuberosum* with other wild species, including late-blight-resistant clones of *S. phureja* and *S. stenotomum.* This gene is shown here to be identical to *Rpi-vnt1.1*. Genotyping shows that *Rpi-vnt1.1* cosegregates with resistance in segregating progeny, and the nucleotide sequence of an ORF amplified using the *Rpi-vnt1.1*-specific primers is identical to *Rpi-vnt1.1.* This level of conservation is surprising, although recent reports that highly conserved and putatively functional *Rpi-blb1* homologs have been found in *S. stoloniferum, S. polytrichon*, and *S. papita* (Vleeshouwers et al. 2008; Wang et al. 2008) and also in *S. verrucosum* (Liu and Halterman 2006) suggest that some *Rpi* genes belong to ancient loci that are conserved through the selection pressure imposed by *P. infestans,* or that genetic exchange (presumably infrequently) can occur between *Solanum* spp. The fact that *Rpi-vnt1.1* and *Rpi-phu1* are identical suggests that the divergence event that led to the two species is relatively recent. This sequence conservation could also be a result of the fact that no other *Rpi-vnt1.1* paralogues are found within the immediate vicinity of *Rpi-vnt1.1*, thereby reducing the opportunity for sequence exchange which is thought to contribute to the evolution of *R* genes and appears to have been the mechanism by which *Rpi-blb1* arose by recombination between two paralogues (*RGA1-blb* and *RGA3-blb*) that reside in the same locus (van der Vossen et al. 2003).

The studies of Jacobs (2008) were not able to assign a species label to the accession CGN18279 due to its occupying conflicting positions within species dendrograms. However, the presence of an *Rpi* gene almost identical to *Rpi-vnt1.1* and the ability of plants from this accession to cross easily with *S. venturii* accessions indicate that CGN18279 is probably *S. venturii.*

It was not possible to identify a BAC clone containing *Rpi-vnt1.2*. This may simply be due to the distribution of BAC clones in the library or it may be that neighboring sequences in the *Rpi-vnt1.2* haplotype cause instabilities within the BAC vector that do not allow it to be propagated in *Escherichia coli.* Due to the lack of a genomic clone of *Rpi-vnt1.2*, we have not been able to demonstrate that this gene confers resistance against *P. infestans* as we have for *Rpi-vnt1.1.* However, a number of lines of evidence lead us to postulate that *Rpi-vnt1.2* is responsible for the resistant phenotype of accession CGN18279. First, mapping data present conclusive evidence that *Rpi-vnt1.2* resides in the same genetic location as *Rpi-vnt1.1.* Second, the resistance proteins differ by only an insertion of 14 amino acids within the CC domain and a further 2 amino acids substitutions within the remainder of the protein; given that only one of these residues is in the LRR region which governs *R* gene specificity (Parniske et al. 1997) and that the substitution is synonymous with respect to physical properties, we expect that this gene is also functional and provides late-blight resistance with the same specificity as *Rpi-vnt1.1*. We also show here that *Rpi-vnt1.3*, the amino acid sequence of which differs from *Rpi-vnt1.2* at a single amino acid residue, maps to an identical region in the resistant *S. venturii* accession CGN18000. Pel and associates (2009) have shown by both *Agrobacterium*-mediated transient assay in *Nicotiana benthamiana* and stable transformation of potato cv. Desiree that this gene is functional and confers resistance against the same spectrum of *P. infestans* isolates as does *Rpi-vnt1.1.*

For a resistance gene to be of use in controlling late blight, it should be demonstrated that the resistance has an element of durability. Although we cannot determine durability of the *Rpi-vnt1.1* gene until it is deployed widely, information from tests against a wide range of isolates in Poland have shown that, between 1999 and 2008, only one isolate (identified in 2008) capable of overcoming *Rpi-phu1* was found (J. Śliwka, *unpublished data*). Importantly, *Rpi-vnt1.1* confers resistance against an aggressive isolate (BPC2006 3928A) of genotype 13, known colloquially as "superblight" or "blue 13", which is prevalent throughout the United Kingdom and is capable of overcoming the durable resistance present in the commercially valuable cv. Stirling and which has been mapped as a major QTL to the R2 locus on chromosome IV (Hein et al. 2007).

To introduce *Rpi* genes into cultivars requires immense efforts by breeders. Potato lines are highly heterozygous out-breeding species and introgression of genes from wild *Solanum* relatives would require recurrent backcrosses to the cultivated parent, which would result in inbreeding depression due to the selfing of the recurrent parent (Bradshaw and Ramsay 2005). Although this can be avoided by using different parents for the backcrossing, doing so would result in the loss of important agronomic and nutritional traits that have been selected at the cost of time and effort. Therefore, the use of genetic modification technologies to transfer single *Rpi* genes into cultivars with combinations of traits that have taken considerable time and financial resources to develop is attractive. Recently, transgenic crops containing the *RB* (*Rpi-blb1*) gene from *S. bulbocastanum* were shown to be highly resistant to *P. infestans* and had no negative effects on tuber size or yield, indicating that the expression of *Rpi* transgenes does not have a fitness cost or cause undesirable physiological traits (Halterman et al. 2008). We propose that the use of genetically uniform lines containing mixtures of *R* genes or, alternatively, mixtures of lines containing single *R* genes isolated from novel *Solanum* spp. is a viable strategy for improving the resistance of current potato cultivars and may provide a means to ensure that valuable *R* genes are not broken.

MATERIALS AND METHODS

Solanum spp. growing conditions.

Seed of *Solanum* accessions (Table 1) was obtained from CGN. Seed was surface sterilized in 70% ethanol for 1 min, disinfected with 1.5% hypochlorite for 5 min, rinsed three times in sterile distilled water, and placed on solid Murashige and Skoog (MS) medium (2% agarose) containing 3% sucrose for germination. Germinated seedlings were transferred to glasshouse facilities and treated regularly with fungicides and pesticides to control thrips, aphids, spider mites, powdery mildew, and early blight (*Alternaria solani*).

P. infestans strains, inoculation, and pathogenicity scoring.

P. infestans isolate 98.170.3 (race 1.3.4.10.11) (Smilde et al. 2005) was provided by D. Shaw at Bangor University, U.K. Isolates 90128, IPO-complex, IPO-0, and EC1 (Table 4) were provided by E. van der Vossen at Plant Research International, Wageningen, The Netherlands. The BPC2006 3928A (superblight, blue 13) isolate was provided by P. Birch, SCRI, Dundee, U.K. and is an isolate currently virulent on a large number of

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commercially grown potato cultivars in the United Kingdom and Europe. Isolates MP324 (Śliwka et al. 2006), MP717, MP778, MP674, MP622, MP618, and MP650 were obtained from IHAR, Poland.

The isolates were maintained at 18°C on Rye B agar (Caten and Jinks 1968). Fresh sporangia were produced in a 2-week cycle by subculturing to fresh plates. Periodically, the ability of isolates to infect host material was confirmed on detached leaves of a suitable, sensitive plant. Mature, fresh sporangia were harvested after 10 days of growth on Rye B medium by flooding the plate with sterile deionized water and allowing the harvested spore suspension to stand for 20 min in a fresh petri dish. After this time, most sporangia are stuck to the plastic surfaces of the dish. Water from the original suspension was replaced by fresh cold water and the sporangia resuspended and incubated at 4°C for 1 to 4 h to induce zoospore release.

A detached leaf assay was used to screen for resistance to *P. infestans* (modified from Vleeshouwers et al. 1999). Two leaves per plant were detached and placed onto moistened tissue paper in a 25-by-25-cm assay plate (Nunc, Rochester, NY, U.S.A.) (six genotypes per plate). Leaves were inoculated with 10-µl droplets of a zoospore suspension (50,000 zoospores ml^{-1}). Inoculated leaves were incubated for 7 to 12 days under controlled environmental conditions (16°C, 16 h of light and 8 h of dark) before scoring phenotypes. Plants with leaves showing sporulating lesions were scored as susceptible; plants with leaves showing no visible symptoms or limited necrosis in the absence of sporulation were scored as resistant. For unclear phenotypes, at least three independent inoculations were carried out. For clear phenotypes (either both leaves resistant or both sensitive), two independent rounds of inoculations were considered sufficient.

DNA isolation and sequencing.

DNA was isolated from plant material using the protocol of Park and associates (2005b). BAC clone DNA was isolated using the Qiagen Midi Prep Kit (Qiagen, Hilden, Germany). BAC ends and PCR products were sequenced using the ABI PRISM Big Dye (v. 3.1) Terminator Cycle Sequencing Ready Reaction kit (PE Applied Biosystems, Foster City, CA, U.S.A.) according to the manufacturer's instructions.

AFLP and SSR analysis and PCR-based mapping.

AFLP was performed essentially as described by Thomas and associates (1995) and Vos and associates (1995) on *Pst*I/*Mse*I-digested template DNA using a preamplification step with *Pst*I+0 and *Mse*I+1 primers and a selective amplification step using *Pst*I+2 and *Mse*I+3 primers. Informative AFLP bands were cut from the gel and rehydrated in TE (10 mM Tris-HCl, pH 8.0, and 0.1 mM EDTA). The gel slices were then transferred to fresh TE and crushed, and the debris removed by centrifugation at 14,000 × *g* for 1 min. For cloning, AFLP fragments were first reamplified by PCR using 2 µl of the supernatant and the same primers as for the original amplification and cloned into pGEM-T Easy (Promega Corp.).

SSR PCR reactions were done in 25-µl reaction volumes containing 20 mM Tris-HCl (pH 8.4); 50 mM KCl; 2.5 mM $MgCl_2$; 0.4 mM each dCTP, dTTP, and dGTP; 0.012 mM nonlabeled dATP; 370 kBq (γ-^{33}P)dATP (Amersham Biosciences, Bucks., U.K.); 0.4 µM each primer; 1 U of *Taq* DNA polymerase (Invitrogen, Carlsbad, CA, U.S.A.); and 100 ng of template DNA. Thermal cycling conditions consisted of an initial denaturation step at 94°C for 4 min, followed by a primer annealing step (either 50 or 55°C depending upon the primer pair used) (Table 3) for 2 min, and an extension step at 72°C for 90s. Subsequent cycles were as follows: 29 cycles of 94°C for 1 min, primer annealing temperature for 2 min, 72°C for 90 s, followed by a final extension step of 72°C for 5 min. Amplification products were denatured by the addition of an equal amount of stop solution (95% formamide containing bromophenol blue and xylene cyanol) and heated to 98°C for 10 min. The reaction (2 to 5 µl) was run on 6% denaturing polyacrylamide gels containing 6 M urea at 100 W for 2 to 4 h. Gels were dried and exposed to X-ray film as for AFLP reactions.

Conventional PCR assays were done in 15-µl reaction volumes containing 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 200 µM each dNTP, 0.4 µM each primer, 0.5 U of *Taq* polymerase, and 10 to 100 ng of template DNA. Thermal cycling conditions typically consisted of an initial denaturation step of 94°C for 2 min followed by 35 cycles of 94°C for 15 s, primer annealing temperature (Table 3) for 30 s, 72°C for 1 min/kb of amplified product, followed by a final extension step of 72°C for 10 min. For sequencing, primers and dNTPs were removed from PCR products by incubation with 0.1 U μl^{-1} exonuclease I and 0.1 U μl^{-1} shrimp alkaline phosphatase at 37°C for 30 min followed by incubation at 80°C for 20 min to denature the enzymes. Long-range PCR reactions were done using Phusion High Fidelity DNA polymerase (Finnzymes Oy, Espoo, Finland) according to the manufacturer's protocol.

Construction of an *S. venturii* BAC library containing *Rpi-vnt1.1* and *Rpi-vnt1.2*.

The *S. venturii* plant K39 which was used to construct the BAC library is a transheterozygote carrying both *Rpi-vnt1.1*, originally from the resistant parent plant A618 (accession CGN18108), and *Rpi-vnt1.2*, from the resistant plant A624 (accession CGN18279).

Plant material was grown on MS medium without sucrose in vitro and young leaf tissue was harvested and stored at –80°C. Frozen leaf tissue (20 g) was used to prepare DNA plugs containing high molecular weight DNA using a method slightly modified from Liu and Whittier (1994) and Chalhoub and associates (2004). The DNA plugs were prepared in 0.7% InCert agarose (Biozym, Oldendorf, Germany), washed in lysis buffer solution (1% sodium lauryl sarcosine, proteinase K at 0.2 mg/ml, and sodium diethyldithiocarbamate at 3.8 mg/ml dissolved in 0.5 M EDTA, pH 8.5) and stored at 4°C in 0.5 M EDTA until required. The stored plugs were soaked in TE buffer, chopped into small pieces, and partially digested with 5 units of *Hin*dIII for 1 h to generate DNA fragments of 50 to 300 kb.

Triple size selection was used to improve the size and uniformity of the inserts as described by Chalhoub and associates (2004). The first size selection was performed on 1% Seakem LE agarose (Biozym) using clamped homogeneous electric field pulsed-field gel electrophoresis (Bio-Rad, Hercules, CA, U.S.A.) at 1 to 40 s, 120°C, 16 h, and 200 V in 0.25× Tris-borate-EDTA buffer directly followed by the second size selection in the same gel at 4 to 5 s, 120°C, 6 h, and 180 V in the same buffer. Agarose gel slices containing partially digested DNA between 100 and 200 kb were excised and divided into two. For the third size selection, the excised gel slices were separately run on 1% Sea Plaque GTG low-melting point agarose (Biozym) at 3 to 4.5 s, 120°C, 14 h, and 180 V. Size-selected DNA fragments were excised from the gel and stored at 4°C in 0.5 M EDTA (pH 8). DNA was recovered in 40 µl 1× Tris-acetate-EDTA buffer by electroelution using a Bio-Rad Electro-elution system (Bio-Rad).

The total eluted DNA was ligated in a 100-µl reaction with 10 ng pIndigoBAC-5 vector (EpiCentre Biotechnologies, Madison, WI, U.S.A.) and 800 U of T4 DNA ligase (New England Biolabs, Ipswich, MA, U.S.A.). The ligation was dialyzed against 0.5× TE buffer for 3 h using Millipore membrane (Millipore, Billerica, MA, U.S.A.). Dialyzed ligation (3 µl) was used to

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transform DH10β electrocompetent cells (Invitrogen, Paisley, U.K.) by electroporation. The BAC library was picked into 274 384-well microtiter plates (Genetix Ltd., Dorset, U.K.).

Transformation of *S. tuberosum* cv. Desiree with *Rpi-vnt1.1*.

A 4.3-kb fragment carrying the *Rpi-vnt1.1* promoter, ORF, and terminator was amplified by PCR using the primers vnt1long-F and vnt1long-R (Table 3) from the BAC clone K39_266I9. This fragment was cloned into pGEM-T Easy (Promega Corp.) and sequenced to confirm that no mistakes had been introduced during PCR. The resulting plasmid was digested with *Eco*RI and the fragment containing the original gene cloned into the *Eco*RI site of pBin19. The resulting plasmid was named pSLJ21152. Plasmid pSLJ21152 was introduced into *Agrobacterium tumefaciens* AGL1.

Transformation of potato cv. Desiree was carried out as described by Kumar and associates (1996). Transformation of tomato cv. Moneymaker was carried out as described by Fillatti et al. (1987). Transgenic plants capable of growth on kanamycin were weaned out of tissue culture into sterile peat blocks before being transplanted to the glasshouse. PCR using primers vnt1ORF-F and vnt1ORF-R, which amplify the full-length *Rpi-vnt1.1* ORF (Table 3), was used to determine whether the kanamycin-resistant plants also harbored the *Rpi-vnt1.1* transgene.

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Identification of RFLP markers closely linked to the *H1* gene conferring resistance to *Globodera rostochiensis* in potato

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Summary. Resistance to the root cyst nematode *Globodera rostochiensis* is an agronomic trait that is at present incorporated into most new potato varieties. Major dominant genes are available that originate from wild and cultivated *Solanum* species closely related to the cultivated European potato (*Solanum tuberosum* ssp. *tuberosum*). One of those genes, *H1*, from *S. tuberosum* ssp. *andigena*, was mapped to a distal position on potato chromosome V using restriction fragment length polymorphism (RFLP) markers. The *H1* locus segregates independently from *Gro1*, a second dominant gene presumably from *S. spegazzinii* that confers resistance to *G. rostochiensis* and which has been mapped to chromosome VII. One marker, *CP113*, was linked without recombination to the *H1* locus.

Key words: Potato – *G. rostochiensis* – RFLP – Marker

Introduction

In potato the gene *H1* confers complete resistance to the root cyst nematode *Globodera rostochiensis* (Woll.) Behrens, pathotypes Ro1 and Ro4 (Kort et al. 1977). The gene was discovered in the Commonwealth Potato Collection (CPC) in 5 accessions (out of about 1300 tested) of *Solanum tuberosum* ssp. *andigena* (Ellenby 1954). Genetic analysis using selfed seeds of accession CPC 1673 revealed the presence of a single dominant gene named *H1* (Toxopeus and Huijsman 1953; Huijsman 1955). The germ plasm of CPC 1673 was subsequently incorporated into *S. tuberosum* ssp. *tuberosum* breeding lines and into many European varieties, among those cvs 'Granola', 'Maris Piper' and others widely cultivated (Ross 1986). The availability of *H1*, together with a few other genes originating from *S. vernei* and *S. spegazzinii*, was sufficient to solve the problem of infection caused by pathotype Ro1 of *G. rostochiensis* (Ross 1986).

The genetic relationship among the available genes conferring resistance to *G. rostochiensis* are to a large extent unknown. Moreover, the identity of resistance genes fom different sources is often unclear, as identification is based on their activity against different pathotypes of the nematode, and these pathotypes are themselves difficult to define and subject to change. Recently, we have mapped a major dominant gene conferring resistance to *G. rostochiensis* to chromosome VII of potato (Barone et al. 1990). This locus was named *Gro1* and, based on pedigree information and resistance tests with different *G. rostochiensis* pathotypes, should correspond to the *Fb* gene of *S. spegazzinii* first described by Ross (1962). In the present article we report the genomic position of *H1* as mapped in a genetic background where *H1* can be traced back to the founder clone CPC 1673.

Materials and methods

The *H1* gene was mapped in the progeny of a cross between the dihaploid line Amaryl H5 carrying *H1* and a 2n *Solanum phureja* line (L85DS3.1) susceptible to the nematode. Amaryl H5 was obtained at the INRA Station de Recherches sur la Pomme de Terre et les Plantes à Bulbe, INRA Kéraiber, Ploudaniel 29260 Lesneven, France, from the 4n variety 'Amaryl' via parthenogenesis after pollination with *S. phureja* (Hermsen and Verdenius 1973). 'Amaryl' had been selected from the cross 'Saskia' × (CPC 1673-20 × Furore) (Joosten 1988). CPC 1673-20 was a resistant plant (genotype *HHhh*) originating from the selfing of *S. tuberosum* ssp. *andigena* accession CPC 1673 (Toxopeus and Huijsman 1953).

Fifty-three tetraploid varieties were obtained as described in Görg et al. (1992). Twenty varieties were selected either for their

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resistance to pathotypes Ro1 and Ro4 of *G. rostochiensis* (beschreibende Sortenliste Kartoffeln 1989) or for containing the *H1* gene (Ross 1986). These varieties were: 'Accent', 'Arnika', 'Barbara', 'Certo', 'Clarissa', 'Darwina', 'Granola', 'Ilse', 'Indira', 'Katja', 'Margit', 'Monza', 'Nora', 'Optima', 'Pamir', 'Panda', 'Pirola', 'Ponto', 'Producent', and 'Quarta'. Thirty-three susceptible varieties were also selected: 'Achat', 'Astrid', 'Atica', 'Carola', 'Cosima', 'Clivia', 'Datura', 'Desirée', 'Erna', 'Erntestolz', 'Fausta', 'Forelle', 'Grandifolia', 'Grata', 'Hansa', 'Hela', 'Ilona', 'Irmgard', 'Isna', 'Isola', 'Linda', 'Luna', 'Mentor', 'Moni', 'Ostara', 'Palma', 'Prima', 'Saphir', 'Saskia', 'Selma', 'Sieglinde', 'Sommerstärke' and 'Ulla'.

Resistance to the *G. rostochiensis* Ro1 population Ecosse was tested in a petri dish according to the method of Mugniéry and Person (1976). Cuttings from plants of the first tuber generation (Mugniéry and Balandras 1986) were inoculated with five juveniles per root. A root system showing the presence of nematode females was rated as susceptible. Resistant genotypes showing the absence of cysts were transferred into pots with infested soil and examined after 3 months to confirm their resistance rating.

Between 0.3 and 0.5 g freeze-dried leaf material per genotype was used for extracting total genomic DNA. DNA extraction, restriction digests, electrophoresis, blotting and hybridization methods have been described previously (Gebhardt et al. 1989). Linkage analysis was performed as in previous investigations (Ritter et al. 1990; Barone et al. 1990). RFLP markers selected to cover all potato chromosomes (Gebhardt et al. 1991; unpublished results from this laboratory) were hybridized to filters with *Taq*I- or *Rsa*I-restricted DNA of the parents and F_1 progeny.

Results

One hundred and eleven hybrid lines of the cross Amaryl H5 × *S. phureja* were tested for resistance to *G. rostochiensis*, pathotype Ro1. Amaryl H5 was totally resistant to Ro1, while the *S. phureja* parent was susceptible. Among the seedlings, 58 were found to be resistant and 53 susceptible which supports the hypothesis that the *H1* gene is heterozygous in Amaryl H5 and segregates in the cross in a 1:1 ratio ($P > 0.5$).

Out of the 111 hybrid lines scored for resistance, for practical reasons only 91 lines, 44 resistant and 47 susceptible, were screened for RFLP alleles that cosegregated with the resistance trait. Linkage was detected with six out of ten informative markers of potato chromosome V: *GP22, GP265, GP270, CP113, GP78* and *GP188*. One marker, *CP113*, was linked with zero percent recombination to the *H1* locus. The linkage map of potato chromosome V, including the position of the *H1* locus as deduced from recombination values measured in the resistant parent Amaryl H5, is shown in Fig. 1. No distortion of segregation ratios was observed for chromosome V in the cross studied. The length of the linkage map of chromosome V of Amaryl H5 was 93 centiMorgans, which is a 20% increase over its length of 78 centiMorgans reported in the RFLP map of Gebhardt et al. (1991).

Fifty-three tetraploid German potato varieties were surveyed for the presence of the RFLP allele that had been found to be totally linked in coupling to *H1* in Amaryl H5. Their names are listed in the Materials and methods. Twenty varieties are described as being resistant to pathotypes Ro1 and Ro4 of *G. rostochiensis* in the Beschreibende Sortenliste Kartoffeln (1989) and in Ross (1986). The RFLP allele indicative of the *H1* gene in Amaryl H5 was, however, not present in the germ plasm surveyed (data not shown). There was also no alternative RFLP allele detectable (with the same restriction enzyme and probe) by which resistant and susceptible varieties could be separated.

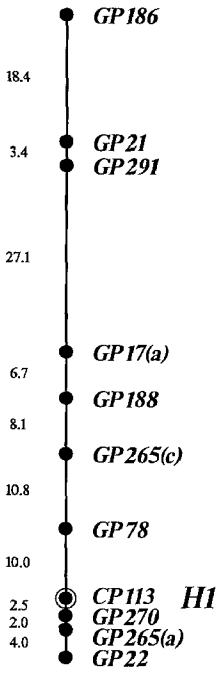


Fig. 1. Linkage map of potato chromosome V as deduced from recombination frequencies in Amaryl H5 carrying the *H1* gene. Map distances are given in centiMorgans (Kosambi 1944). *GP* markers are derived from genomic DNA; *CP* markers from the cDNA of potato. *Small letters in parenthesis* indicate that more than one locus was mapped with the same marker probe. The cDNA marker CP113 is linked with zero percent recombination to the *H1* locus

Discussion

The potential of RFLPs for marker-based selection was recognized 10 years ago (Beckmann and Soller 1983; Tanksley 1983). A prerequisite for using RFLP markers is that ones have to be identified that are closely linked to agronomically relevant genes. Resistance to the root cyst nematodes *G. rostochiensis* and *G. pallida* are considered to be important traits in breeding programs. In this article we report a mapping experiment that identifies four RFLP markers linked, within 10 centiMorgans, to the *H1* gene conferring resistance to *G. rostochiensis*. One marker, *CP113*, was shown to be linked to the *H1* locus without recombination, based on an F_1 population of 91 genotypes. The *H1* locus maps at the end of potato

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chromosome V and, therefore, segregates independently from the nematode resistance locus *Gro1*, which has been mapped onto chromosome VII (Barone et al. 1990). *H1* is also genetically unrelated to the *Mi* locus that induces resistance to the root knot nematode *Meloidogyne incognita*; this locus is located on chromosome VI of the homeologous tomato genome (Klein-Lankhorst et al. 1991; Messeguer et al. 1991). Two other resistance loci have also been allocated to chromosome V: the *Rx2* locus conferring extreme resistance to potato virus X (Ritter et al. 1991) and *R1* conferring race-specific resistance to *Phytophthora infestans* (Leonards-Schippers et al. 1992). Both of these latter loci map close to the marker locus *GP21* and, therefore, are located in a segment of chromosome V that is 63 centiMorgans away from *CP113* (Fig. 1). If we also take *Pto*, a *Pseudomonas* resistance gene identified on tomato chromosome V (Martin et al. 1991), into consideration and if we assume homeology between the potato and tomato genome for resistance loci, chromosome V appears to be particularly rich in disease resistance loci.

There may be several reasons for the absence of the RFLP allele linked to *H1* in Amaryl H5 in a series of resistant potato varieties having reported pathotype specificity of *H1*. First, and this seems the most likely explanation, the source of resistance in the varieties tested was not the same as in Amaryl H5. Genetic evidence, for example, indicated that resistant germ plasm derived from *S. vernei* in some cases contains the same genetic locus as that present in *S. tuberosum* ssp. *andigena* CPC 1673 (Scurrah et al. 1973). *S. vernei*, in addition to CPC 1673, has also been widely used in potato breeding for introducing resistance to *G. rostochiensis*. Second, in the *S. tuberosum* ssp. *andigena* germ plasm from which the *H1* gene originated, more than one *H1* allele was present in a polymorphic genetic background. Third, during extended backcross breeding the linkage between *H1* and closely linked marker sequences have been broken. To be able to use maker *CP113* for selecting *H1* genotypes, it is therefore necessary – as in human genetics – to determine the informative allele or the marker phase in the germ plasm employed in crosses.

A positive and important outcome of this study is that the selection of *Gro1, H1* genotypes is now feasible based on the RFLP markers flanking both loci. This should result in phenotypes having highly durable resistance to most, if not all pathotypes of *G. rostochiensis*.

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Mapping and marker-assisted selection for a gene for extreme resistance to potato virus Y

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Abstract The chromosomal location of the major gene Ry_{adg} controlling extreme resistance to potato virus Y (PVY) in *Solanum tuberosum* subsp. *andigena* was identified by RFLP analysis of a diploid potato population. A total of 64 tomato and potato RFLP markers were screened with the bulked segregant analysis (BSA) on segregants extremely resistant, hypersensitive or susceptible to PVY. Four markers TG508, GP125, CD17 and CT168 at the proximal end of chromosome XI showed close linkage with extremely resistant phenotypes. TG508 was identified as the closest marker linked with the Ry_{adg} locus with the maximum map distance estimated as 2.0 cM. The 4 markers linked with the Ry_{adg} locus were tested on independent tetraploid and diploid potato clones and were subsequently found useful for marker-assisted selection for plants containing Ry_{adg}.

Key words Potato virus Y · Resistance gene · *Solanum tuberosum* subsp. *andigena* · Potato · RFLP

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Introduction

Potato Y potyvirus PVY is globally one of the most important viral pathogens of the cultivated potato (*Solanum tuberosum* L.) and can reduce yields up to 80% (Hooker 1981). PVY can be controlled using virus-tested seed potatoes. However, PVY-infected volunteer potato plants or weeds usually exist in the proximity of the potato crop and dispersal of PVY by aphids frequently occurs to potatoes in the field. Therefore, resistance to PVY in potato cultivars provides the most effective control against this virus (de Bokx and van der Want 1987).

Two major types of monogenically inherited resistance to PVY are known in cultivated and wild potato species (*Solanum* spp.), namely hypersensitive resistance (H) and extreme resistance (E) (Ross 1986). H controlled by the *Ny* genes is often PVY strain group-specific, whereas E controlled by the *Ry* genes is effective against all strains of PVY (Cockerham 1970; Jones 1990). Following infection with PVY, potato plants expressing *Ny* develop necrotic lesions in infected leaves and/or necrosis in systemically infected parts. Plants expressing *Ry* remain symptomless, except limited necrosis may develop in the systemically infected leaves in a few genotypes following graft-inoculation. No PVY titers detectable with ELISA develop in inoculated plants expressing *Ry* (Ross 1986).

The *Ry* genes known in the cultivated and wild potato species and utilized in potato breeding programs include Ry_{sto} in *S. stoloniferum* Schlechtd. et Bche (Ross 1958; 1986)

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and Ry_{adg} in *S. tuberosum* subsp. *andigena* Hawkes (Muñoz et al. 1975). So far, no gene for resistance to PVY has been localized genetically. Resistance for PVY in *S.t. andigena* was previously expected to function at a single locus. However, a few progeny hypersensitive to PVY were observed among extremely resistant and susceptible progeny in the crossing populations of *S.t. andigena*. Thus, Ry_{adg} was thought to follow a 'distorted segregation' (Muñoz et al. 1975; Gálvez et al. 1992). Recently, Valkonen et al. (1994b) found evidence for two resistance genes (Ry_{adg} and Ny_{adg}) in *S.t. andigena*. The expression of Ry_{adg} was found to be epistatic to the expression of Ny_{adg} and consequently, the mapping of the PVY resistance loci in *S.t. andigena* became attractive as the segregation of resistance genes corresponding to discrete phenotypes was expected.

In this study, the linkage maps of potato and tomato (*Lycopersicon esculentum* Mill.) based on restriction fragment length polymorphism (RFLP) (Tanksley et al. 1992) were utilized to identify RFLP markers linked to Ry_{adg} in a diploid potato progeny. The significant markers were applied for marker-assisted selection of extreme resistance to PVY controlled by Ry_{adg} in diploid and tetraploid potatoes.

Materials and methods

Plant material

A (di)haploid (2n=2x=24) potato population (F_1) was produced via a sexual cross between the haploid (2n=2x=24) potato breeding line 2x(V-2)7 containing Ry_{adg} (extremely resistant to PVY) and the diploid (2n=2x=24) potato clone 84.194.30 (susceptible to PVY) (Valkonen et al. 1994b). A total of 54 progeny of this F_1 population, which had previously been tested for phenotypic resistance responses to a PVY isolate belonging to the ordinary strain group of PVY (PVY^O) (Valkonen et al. 1994b), were included in this study for the identification of markers linked to Ry_{adg}. The markers were further tested for selection of PVY resistance using tetraploid potato cultivars and tetraploid and diploid potato breeding lines with different genetic backgrounds (Table 1) obtained from the International Potato Cen-

Table 1 Examination of tetraploid potato cultivars and tetraploid and diploid potato breeding lines using the RFLP markers TG508, GP125, CD17 and CT168 which detected a single band unique for the genotypes carrying the gene Ry_{adg} in the diploid mapping population of this study

Clone	Resistance donor species[a]	Resistance		Presence of the marker band[c]			
		Resistance expression	Reference	TG508	GP125	CD17	CT168
Tetraploid (2n = 2x = 48)							
Allegany	*adg*	H	Valkonen et al. 1994a	–	–	–	–
Andover		S	Anonymous 1994	–	–	–	–
Atlantic		S	Valkonen et al. 1994a	–	–	–	–
Belrus		S	S. A. Slack, unpublished	–	–	nd	nd
Desiree	*tbr*	H	Jones 1990	–	–	nd	nd
Katahdin	*tbr*	H	Jones 1990	–	–	nd	nd
Monona		S	Anonymous 1994	–	–	nd	nd
Norchip		S	Anonymous 1994	–	–	nd	nd
Pentland Ivory	*tbr*	H	Jones 1990	–	–	–	–
Pito	*tbr*	H	Valkonen and Mäkäräinen 1993	–	–	–	–
Sebago	*tbr*	H	This study	–	–	nd	nd
Superior		S	Anonymous 1994	–	–	–	–
NY99		S	R. L. Plaisted, unpublished	–	–	–	–
954.3CA		S	Watanabe et al. 1994a	–	–	–	–
TA.1.27.1.1		S	Watanabe et al. 1992	–	–	nd	nd
E74-7	*adg*	E	This study	+	+	+	+
N140-201	*adg*	E	This study	+	+	+	+
Q237-8	*adg*	E	R. L. Plaisted, unpublished	+	+	+	+
TA3.5.3.6	*adg*	E	Watanabe et al. 1992	+	+	+	+
TA3.5.3.7	*adg*	E	Watanabe et al. 1992	+	+	+	+
TA3.8.3.2	*adg*	E	Watanabe et al. 1992	+	+	nd	nd
TA3.8.3.3	*adg*	E	Watanabe et al. 1992	+	+	nd	nd
TA3.8.3.4	*adg*	E	Watanabe et al. 1992	+	+	nd	nd
Diploid (2n = 2x = 24)							
2x(v-2)7	*adg*	E	Valkonen et al. 1994b	+	+	+	+
2x(v-3)30	*sto*	E	Watanabe et al. 1994b	+	+	–	–
86.61.26	*sto*	E	Valkonen et al. 1994b	+	–	–	–
IvP 35	*phu*	E	Valkonen et al. 1995	–	–	–	–
CPC 2451	*brd*	E	Valkonen et al. 1995	–	–	–	–
84.36.29		S	Watanabe et al. 1994b	–	–	nd	nd
84.194.30		S	Valkonen et al. 1994b	–	–	–	–
J-40		S	Watanabe et al. 1994b	–	–	nd	nd

[a] *adg*, *Solanum tuberosum* subsp. *andigena*; *sto*, *S. stoloniferum*; *phu*, *Solanum phureja*; *brd*, *Solanum brevidens*
[b] E, Extremely resistant; H, hypersensitive; S, susceptible
[c] Marker band detected (+) or not detected (–); nd, not determined

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ter (CIP), Lima, Peru; Department of Plant Breeding and Biometry, Cornell University, Ithaca, N.Y., USA; and Cornell Foundation Uihlein Seed Potato Farm, Lake Placid, N.Y., USA. In some of these potato genotypes, resistance to PVY was tested in this study by graft-inoculation with a previously described isolate of PVY^O (Valkonen and Mäkäräinen 1993) under natural daylight in the greenhouse (means of the minimum and maximum temperatures – 19°C and 24°C).

RFLP analysis

DNA was extracted from plants grown in the greenhouse as described by Bernatzky and Tanksley (1986) except that sodium bisulfite (5 g/l) was used instead of mercaptoethanol. RFLP between the progeny was studied using the bulked segregant analysis (BSA) (Michelmore et al. 1991). DNA samples of the progeny were bulked to three pools based on the resistance phenotype (33 progeny with E, 7 progeny with H and 14 progeny susceptible (s) to PVY). Bulked DNA of the progeny and the DNA samples of the parental clones were digested with *Dra*I, *Eco*RI, *Eco*RV, *Hin*dIII or *Xba*I (Boehringer Mannheim), and 10 μg of the digested DNA was loaded and separated on a 0.8% agarose gel using electrophoresis. Southern blotting was carried out as described by Sambrook et al. (1989).

A total of 64 tomato and potato genomic and cDNA clones were used as probes (Gebhardt et al. 1989; Tanksley et al. 1992). These selected markers span the entire potato genome and were located at 20-cM intervals. Probes were labelled with digoxigenin or [^{32}P] and hybridized to potato DNA on Southern blots; the signal was detected by chemiluminescence following the manufacturer's instructions (Boehringer Mannheim) or by autoradiography. The putatively polymorphic markers identified based on BSA were hybridized to filters containing DNA of the progeny individuals and the parental lines, each loaded separately.

Linkage analysis was performed using the MAPMAKER/EXP 3.0 software (Whitehead Institute, Cambridge, Mass., USA). Extreme resistance was treated as an extra RFLP fragment being present in the extremely resistant genotypes and absent in the susceptible and hypersensitive genotypes.

Results

Polymorphism between the DNA pools of the extremely resistant, hypersensitive and susceptible progeny was initially detected with TG508 following digestion with *Eco*RI (Fig. 1). Flanking markers close to TG508 in chromosome XI were then used for hybridization, and polymorphism between the three pools was detected with the markers CD17 (*Hin*dIII digest), CT168 (*Dra*I digest) and GP125 (*Eco*RV digest). With all the markers, a single band of 6–8 kb was identified which was unique only to the E phenotype pool and not observed in the pools of the H and S phenotypes. Hybridization of these 4 putatively significant markers against filters containing DNA of the progeny individuals (Fig. 2) showed that all were linked to Ry_{adg} according to the test for independent assortment (TG508: χ^2=51.00; CT168: χ^2=34.38; GP125: χ^2=46.17; CD17: χ^2=39.27; P<0.005). The closest linkage to Ry_{adg} was identified for TG508 (maximum map distance estimated as 2.0 cM). Thus, the data indicated that Ry_{adg} was located at the proximal region of chromosome XI (Fig. 3).

The applicability of CD17, GP125 (Fig. 4), CT168 and TG508 as markers for E to PVY was tested in selected tetraploid potato cultivars and diploid and tetraploid potato breeding lines (Table 1). All markers gave a signal of the correct size in the potato genotypes carrying Ry_{adg}. Additionally, TG508 gave a positive signal in diploid breeding lines 86.61.26 and 2x(V-3)30, which carry the gene Ry_{sto}. GP125 also showed a positive signal in 2x(V-3)30. In contrast, no signal band of the correct size was detected by any marker in *S. phureja* IvP35 and *S. brevidens* CPC 2451, both of which also express E to PVY. Similarly, most of the potato genotypes expressing necrosis (H) following PVY infection were not detected with the markers, except for potato breeding lines N140-201 and E74-7, which showed a positive signal with all four markers (Fig. 4, Table 1). In the test for PVY resistance by graft-inoculation, N140-201 and E74-7 showed necrotic streaks in minor veins, and E74-7 also showed small chlorotic spots with a necrotic centre, but no PVY was detected by ELISA. Thus,

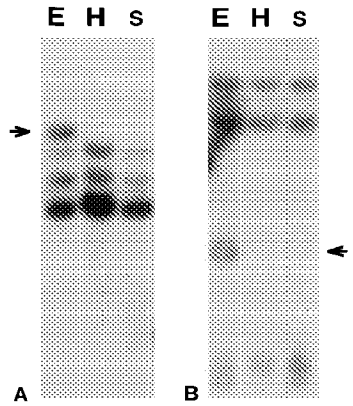


Fig. 1A, B Bulked segregant analysis of diploid potato progeny pooled on the basis of resistance to PVY^O (*E* extreme resistance, *H* hypersensitive resistance, *s* susceptible). DNA pools were digested with *Xba*I (**A**) and *Eco*RI (**B**) and probed with TG508. *Arrowhead* indicates the marker band specific to the extremely resistant progeny carrying Ry_{adg}



Fig. 2 Hybridization with the marker CD17 to *Hin*dIII-digested individual progeny obtained from the cross 2x(v-2)7 (P1) 84.194.30 (P2). *P1* carries the gene Ry_{adg} for extreme resistance (*E*) and the gene Ny_{adg} for hypersensitive resistance (*H*) to PVY, whereas *P2* is susceptible (*S*) to PVY. The marker band specific to the E genotypes is indicated by an *arrowhead*. Note the two recombinants (marked with *) detected with CD17

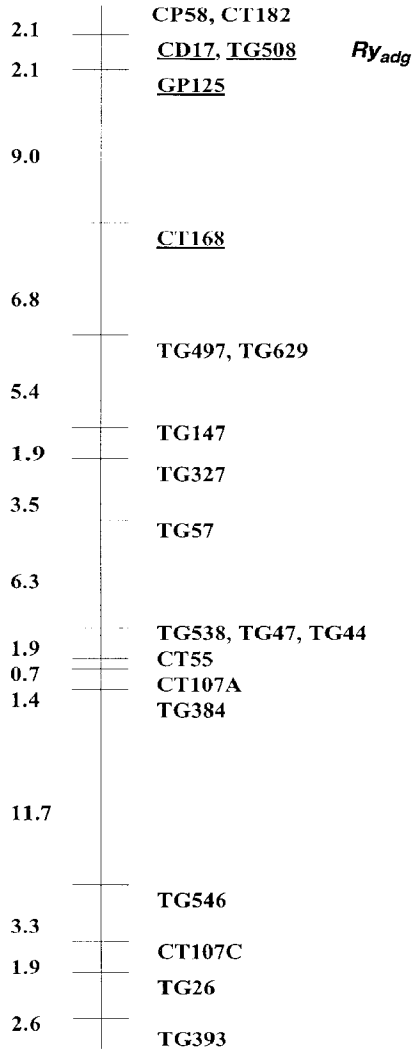


Fig. 3 Composite genetic map of potato chromosome XI showing the predicted position of Ry_{adg}. Map distances are based on segregation in an interspecific cross of diploid potatoes (Tanksley et al. 1992) except for the *underlined* markers, which correspond to linkage analysis of the diploid progeny of this study (LOD>3.0)

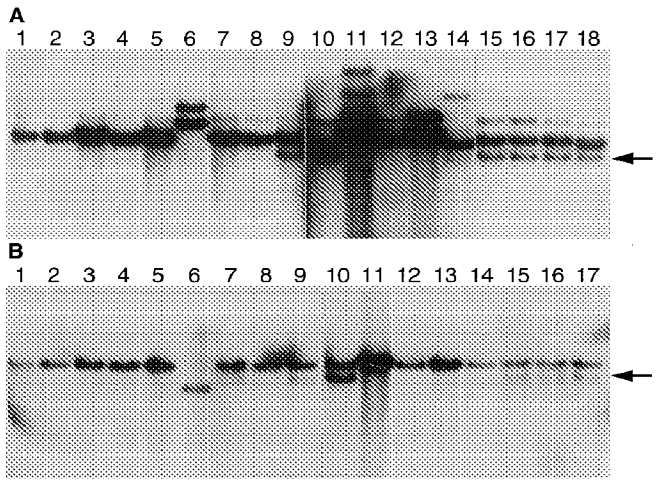


Fig. 4A–B Hybridization with the marker GP125 (**A**) to *Eco*RI-digested and with the marker CD17 (**B**) to the *Hin*dIII-digested DNA of diploid and tetraploid potato breeding lines which are extremely resistant (*E*), hypersensitive (*H*) or susceptible (*s*) to PVY (for detailed description, see Table 1. *Lane 1*, Atlantic (s), *2* Andover (s), *3* NY99 (s), *4* Pentland Ivory (H), *5* Bintje (s), *6 S. brevidens* CPC2451 (E), *7* Allegany (H), *8* Pito (H), *9* E74-7 (H), *10* 2x(v-2)7 (E), *11* 81.61.26 (E), *12* 84.194.30 (s), *13 S. phureja* IvP35 (E), *14* 2x(v-3)30 (E), *15* TA3.5.3.6 (E), *16* TA3.8.3.3 (E), *17* TA3.5.3.7 (E), *18* N140-201 (E)

sensu Ross (1986), both clones were extremely resistant to PVY despite the necrotic symptoms which developed (in contrast, e.g., 2x(V-2)7 carries Ry_{adg} and develops no symptoms following graft-inoculation with PVY; Valkonen et al. 1994b).The marker bands were not observed in any genotype susceptible to PVY.

Discussion

Earlier genetic studies on E and H to PVY in potato species have indicated that these resistances are controlled by single dominant genes (Cockerham 1970; Ross 1986). *S.t. andigena* has been utilized as a source of E to PVY in breeding programs mainly in North and South America (Muñoz et al. 1975; Gálvez et al. 1992). Crosses with *S.t. andigena* have resulted in a few progeny hypersensitive to PVY among the extremely resistant and susceptible progeny. This has been explained as a 'distorted segregation' as the resistance was expected to function at a single locus (Galvez et al. 1992). A recent study by Valkonen et al. (1994b) showed that *S.t. andigena* has two resistance genes, namely Ry_{adg} controlling E to PVY^O and Ny_{adg} controlling H to PVY^O, and that Ry_{adg} is epistatic to Ny_{adg}. Due to epistasis, the genotypes carrying both Ry_{adg} and Ny_{adg} express E to PVY, which is similar to the resistance of the genotypes carrying Ry_{adg} only. In contrast, the genotypes carrying only Ny_{adg} develop necrotic symptoms (Valkonen et al. 1994b). The 4 RFLP markers linked to Ry_{adg} were not associated with the hypersensitive progeny of our mapping population. These data provided further evidence that Ry_{adg} is functional at a locus different from that of Ny_{adg}.

During recent years, diploid potato populations have been utilized for the identification of chromosomal loci controlling disease or nematode resistance in potato (e.g., Barone et al. 1990; Leonards-Schippers et al. 1992; Gebhardt et al. 1993; Pineda et al. 1993; El-Kharbotly et al. 1994, 1996; Gebhardt 1996; Jacobs et al. 1996). Two loci controlling virus resistance in potato have been identified to date, namely *Rx1* and *Rx2* controlling E to potato X potexvirus on chromosomes V and XII, respectively (Ritter et al. 1991). Genetic mapping of the genes for resistance

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to PVY and other potato viruses using a wider range of potato species and genotypes will permit the identity of virus resistance genes in potato germplasm to be compared. For example, the positive signals obtained with TG508 and GP125 in potato clones carrying Ry_{sto} in this study illustrate the need for further studies to examine whether Ry_{sto} is also located on chromosome XI, whereas the lack of positive signals in *S. phureja* and *S. brevidens* suggests that the PVY resistance gene(s) of these species may be different or at different chromosomal loci. The region of chromosome XI containing the Ry_{adg} locus seems to carry several resistance genes in the solanaceous species. According to Gebhardt (1996, and personal communication), the marker locus *CP58* (see Fig. 3) is linked to the *N* gene for H to tobacco mosaic virus in tobacco (Whitham et al. 1994). A few DNA fragments amplified by the polymerase chain reaction (PCR) from potato genomic DNA using primers designed according to the *N* gene sequence (Whitham et al. 1994) and the *RPS2* gene sequence of *Arabidopsis* (Bent et al. 1994) map to this region of chromosome XI in potato (Leister et al. 1996). This suggests that resistance genes with different specificities may be arranged in tandem arrays on chromosomes as proposed by Staskawicz et al. (1995) and Gebhardt (1996).

This study reports the localization of Ry_{adg} to chromosome XI, which can serve as a starting point for the positional cloning of Ry_{adg}. For this purpose and for fine mapping, saturation of the genetic map by increasing the amount of markers at the chromosomal region of interest is in progress. Two yeast artificial chromosomes of tomato genomic DNA which contain the GP125 fragment and, thus, are likely to correspond to the region of our interest on chromosome XI are being utilized.

All 4 markers showed a positive signal in breeding lines N140-201 and E74-7 (Fig. 4), which contain Neo-Tuberosum (i.e, *S.t. andigena*). However, these breeding lines expressed necrotic symptoms following graft-inoculation with PVY. It is hypothesized that these 2 breeding lines contain Ry_{adg} but that the genes of the parental, PVY-susceptible potato genotypes interfere with the expression of Ry_{adg}, which thereby changes the expression of the symptomless E phenotype towards a necrotic phenotype. This is consistent with the observations of Ross (1958) who concluded that the polygenes of susceptible potatoes converted E towards H in several progeny containing Ry_{sto}. However, he found no evidence that E or H to PVY could be converted to susceptibility to PVY.

The examples mentioned above show that distinct resistance genes (*Ry* and *Ny*) can have a similar phenotypic expression in some cases. Thus, selection for progeny with the gene of interest is not always possible based on symptom expression following virus inoculation, and molecular markers linked to the gene of interest are required. Compared to the classical crossing schemes (Ross 1986), marker-assisted selection (MAS) for resistance can improve the cost-effectiveness and significantly speed up the introgression of resistance genes to potato cultivars as large-scale screening for resistance phenotypes can be avoided (Tanksley et al. 1989; Watanabe 1994). Localization of Ry_{adg} to the proximal end of chromosome XI in this study is the first step in developing a molecular probe to be used for MAS for PVY resistance in potato breeding lines. Four markers were identified which were linked to Ry_{adg} and which could be used to distinguish diploid and tetraploid potato genotypes carrying Ry_{adg}. MAS using the significant markers could be further enhanced by development of a PCR-based immunoassay (Skerritt and Appels 1995).

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The *R3* Resistance to *Phytophthora infestans* in Potato is Conferred by Two Closely Linked *R* Genes with Distinct Specificities

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The *R3* locus of potato (*Solanum tuberosum* L.) confers full resistance to avirulent isolates of *Phytophthora infestans*, the causal agent of late blight. *R3* resides in the distal part of chromosome 11 and segregates in a potato mapping population, from which a well-saturated amplified fragment length polymorphism map is available. Using a population of 1,748 plants, we constructed a high-resolution genetic map at the *R3* locus. Using the combination of fine mapping and accurate disease testing with specific *P. infestans* isolates, we detected that the *R3* locus is composed of two genes with distinct specificities. The two genes *R3a* and *R3b* are 0.4 cM apart and have both been introgressed from *S. demissum*, the 'donor' species of most characterized race-specific *R* genes to *P. infestans*. A natural recombinant between *R3a* and *R3b* was discovered in one accession of *S. demissum*. The synteny between the *R3* locus and the tomato *I2* locus is discussed.

The central dogma of molecular plant pathology is the gene-for-gene model, which states that, for every dominant resistance (*R*) gene in the plant, there is a corresponding dominant avirulence (*Avr*) gene in the pathogen (Flor 1971). The presence of matching gene pairs controls the outcome of the interaction of many pathosystems (Thompson and Burdon 1992), including the interaction between potato (*Solanum tuberosum* L.) and its oomycete pathogen *Phytophthora infestans* (Black et al. 1953; van der Lee et al. 2001). A corollary of the gene-for-gene model is that receptors encoded by *R* genes can directly or indirectly perceive the products of corresponding *Avr* genes, enabling recognition of the pathogen and subsequent elicitation of an array of plant defense responses that eventually lead to resistance (Dangl and Jones 2001; Keen 1990). In many cases, the induced defense is manifested as a hypersensitive response (HR) that is associated to a programmed cell death at the initial site of infection (Morel and Dangl 1997). Unexceptionally, HR was found to be associated with all forms of resistance to *P. infestans*, including race- or cultivar-specific resistance, partial resistance, and nonhost resistance, indicating that recognition occurs independently of the type of resistance (Kamoun et al. 1999; Vleeshouwers et al. 2000). To understand the mechanisms underlying recognition, it is essential to isolate the interacting gene pairs from both the host and the pathogen. For this purpose, single dominant *R* genes and *Avr* genes are preferred because they are genetically more accessible.

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Late blight, caused by *P. infestans*, is the most devastating disease for potato production worldwide (Fry and Goodwin 1997). Resistance conferred by dominant *R* genes is easier to manipulate than quantitative resistance. A number of *R* genes for late blight resistance have been introgressed from wild relatives into cultivated potato through sexual and somatic hybridization. Some of these have been mapped to the potato genome through linkage to specific DNA markers (Gebhardt and Valkonen 2001). Recently, two of them have been cloned. One is *R1*, a race-specific *R* gene originating from *S. demissum* (Ballvora et al. 2002). Another is *RB* or *Rpi-blb1*, which confers broad-spectrum resistance and is derived from *S. bulbocastanum* (Song et al. 2003; van der Vossen et al. 2003). Both genes are members of the *R* gene class predicted to encode receptors with coiled coil (CC), nucleotide binding site (NBS), and leucine-rich repeat (LRR) domains (Ballvora et al. 2002; Song et al. 2003; van der Vossen et al. 2003).

Previously, *R3* was mapped as a single dominant locus on the distal part of the short arm of potato chromosome 11, closely linked to restriction fragment length polymorphism (RFLP) markers GP250A, GP185, and TG105A (El Kharbotly et al. 1994). The short arm of chromosome 11 in potato is syntenic to the long arm of chromosome 11 in tomato (Dong et al. 2000; Tanksley et al. 1992). Like many other *R* loci, *R3* resides in a hot spot for resistance to various pathogens. A number of major *R* loci and quantitative trait loci (QTL) for resistance from Solanaceae crops have also been anchored in the same region (Grube et al. 2000; Pan et al. 2000), including another two *P. infestans* resistance genes, *R6* and *R7* (El Kharbotly et al. 1996). To date, the tomato *I2* gene that confers resistance to *Fusarium oxysporum* f. sp. *lycopersici* is the only *R* gene cloned from this region (Ori et al. 1997; Simons et al. 1998).

In this report, we describe the construction of a high-resolution genetic map of the *R3* locus, using the saturation from the ultrahigh density potato map with over 10,000 amplified fragment length polymorphism (AFLP) markers and the resolution from a large segregating F1 population. Interestingly, two functional *R* genes instead of one were found at the locus, and they displayed a differential reaction to some well-defined *P. infestans* isolates. The occurrence of two *R* genes at the *R3* locus was confirmed in a natural population of *S. demissum*. The colinearity between tomato and potato in this region of chromosome 11 enabled the development of *R3*-flanking markers from tomato sequences and the association of the locus with the *I2* locus of tomato.

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RESULTS

Identification and localization of *R3* in the ultrahigh density map.

R3 was previously mapped on the distal part of chromosome 11 by using a diploid segregating population, J91-6164, which was derived from the cross between Esc.42 (Table 1, *R1r1*, *R3r3*) and 84-1031-29 (1031) (Table 1, *r1r1*, *r3r3*). Esc.42 is a dihaploid derivative from potato cv. Escort (El Kharbotly et al. 1994). SH83-92-488 (SH) (Table 1), the maternal clone of the mapping population of the ultrahigh density map, shares its genetic background with Escort. Pedigree information revealed that both SH and Escort descended from potato cv. Reaal, which carries *R1* and *R3* (Table 1, Mastenbroek 1953).

To verify whether this genetic background includes *R3* so as to enable the utilization of the global coverage with AFLP markers on the ultrahigh density map, we analyzed the resistant clones SH, J91-6164-21 (6164) (Table 1), Reaal, and susceptible clones RH89-039-16 (RH) (Table 1) and 1031 with polymerase chain reaction (PCR) markers GP250, TG105, and STM0025 (Table 2). The clone 6164 is a progeny of the previous mapping population J91-6164 (El Kharbotly et al. 1994). It carries *R3* but not *R1*. RH is the paternal clone of the population of the ultrahigh density map. In J91-6164, GP250 and TG105 mapped as RFLP markers are proximal to *R3* (El Kharbotly et al. 1994). STM0025 is 5 cM distal to GP250 (Milbourne et al. 1998). The band pattern of all three PCR markers suggested that at the *R3* region all three resistant clones carry a genomic fragment that is identical-by-descend (Fig. 1A).

To confirm the presence of *R3* in SH, we characterized SH, 6164, RH, and 1031 for race-specific resistance with a panel of *P. infestans* isolates (Table 3) on both detached leaves and in vitro plantlets. SH and 6164 displayed a typical HR when inoculated with *P. infestans* isolates 89149-9, IPO-0, and H30P04 (Table 3), which were all avirulent to the *R3* differential Mastenbroek *R*3 (Ma*R*3) (Table 1, van der Lee et al. 2001). Both SH and 6164 exhibited full susceptibility to *P. infestans* isolates 89148-27 and 90128 (Table 3), which are both virulent on Ma*R*3. Control clones RH and 1031 were

Table 1. Plant materials used in this study

Clone	Abbreviation	Species	Known *R* Gene	Remark
RH89-039-16	RH	*Solanum tuberosum*	None	Paternal clone of the SH × RH *R3* mapping population
SH83-92-488	SH	*S. tuberosum*	*R3*	Maternal clone of the SH × RH *R3* mapping population
Esc.42	Esc	*S. tuberosum*	*R1, R3*	Paternal clone of the J91-6164 *R3* mapping population[a]; introgression study
84-1031-29	1031	*S. tuberosum*	None	Maternal clone of the J91-6164 *R3* mapping population; introgression study
J91-6164-21	6164	*S. tuberosum*	*R3*	A progeny of J91-6164; introgression study
Reaal	Reaal	*S. tuberosum*	*R1, R3*	Common ancestor of Esc and SH; syn. to 4768-15[b]; introgression study
Mastenbroek *R3*	Ma*R3*	*S. tuberosum*	*R3*	The *R3* differential developed by Mastenbroek; syn. to CEBECO 4642-1[c]
1024-2	1024	*S. tuberosum*	None	Introgression study
CE-10	CE	*S. tuberosum*	None	Introgression study
Katahdin	KA	*S. tuberosum*	None	Introgression study
CGN17810	17810	*S. demissum*	*R1, R2, R3, R4, R7, R8*	One of the *R3* donor accessions; introgression study
CGN18313	18313	*S. demissum*	None[d]	Introgression study

[a] El Kharbotly et al. 1994.
[b] Mastenbroek 1953.
[c] van der Lee et al. 2001.
[d] This study revealed that 18313 carries *R3b*.

Table 2. Polymerase chain reaction (PCR) markers used in this study, listed according to their position on the high-resolution map

Marker	GenBank accession	Bin	Phase[a]	Primer (5'-3')	Temperature (°C)	Fragment size (bp)	Restriction enzyme
St3.3.11	U60083	46	C	GCT AAG CTG GGA TTG TTG TCT TAC TGT GCC ACC CGT TGA G	52	380	a.s.[b]
cLET5E4	AW038480	65/66	C	CCA GGC ATG CTC AAT TTG GAG T TTC CCT GTT TGG ACT ACT TGT GGA	55	310	*Hha*I
St1.1	U60069	65/66	C	GCT CAT TCG ACT TAA AGG TTG TTG GGC AGC TCC CAT ATT TCA CTT CTC	60-52[c]	450	*Hae*III
TG105		65/66	C	TCA CAT GAG CTG GGA GAA AT AAA GGC CTG TTG CTG AGA G	54	650	*Hin*fI
GP250		65/66	C	ACC AGT AGG ACC ACC ACC AAC AAT GAT CGT GAC GGC TCT ACT CTT TTA TGA	60-52[c]	410	*Vsp*I
STM0025		65/66	C	GTT CAT GAT TGT GAA TGC TC ATG ACT CAA CCC CAA ATG	59	170	a.s.
GP185		65/66	C	CTG GTA ATA GTA GTA ATG ATT CTT CGT C TTG TTC AAT GGA GCA CTT GC	54	440	*Bst*UI
TG26		65/66	C	GAG AGG GGA CAC TTT TAT TTA TTC A GAG ATC TTC CCG CCG CTG TG	49	1600	a.s.
CT120		67	R	CGA GGG GGC GAA GGA TT CCA TGA GAT AAA CGA GGA ACC AGT	52	360	*Tsp*509I

[a] Phase to *R3* (C for coupling and R for repulsion).
[b] a.s. = allele specific.
[c] Touchdown PCR with temperature gradually decreasing from 60 to 52°C.

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susceptible to all the five isolates (Fig. 1B). This demonstrated that SH carries an *R* gene (genes) with the same specificity as *R3*.

To test whether the *P. infestans* resistance in SH is inherited as a single *R* gene, we examined the segregation for resistance in the mapping population (SH × RH) of the ultrahigh density map. We inoculated the population with *P. infestans* isolate IPO-0. The distinction between the resistant and the susceptible phenotypes was clear, i.e., the resistant individuals showed a dark localized necrosis (HR) at 3 to 4 days postinoculation (dpi), while the susceptible ones gave extensive sporulation at 5 dpi. This suggested the resistance was controlled by one or more major genes. In a total of 108 individuals tested, 46 were resistant and 62 were susceptible, which fitted the Mendelian 1:1 ratio. Although the segregation slightly skewed toward susceptibility, the ratio supported the inheritance of a single dominant *R* gene ($\chi^2 = 2.37$; $p \geq 0.1$).

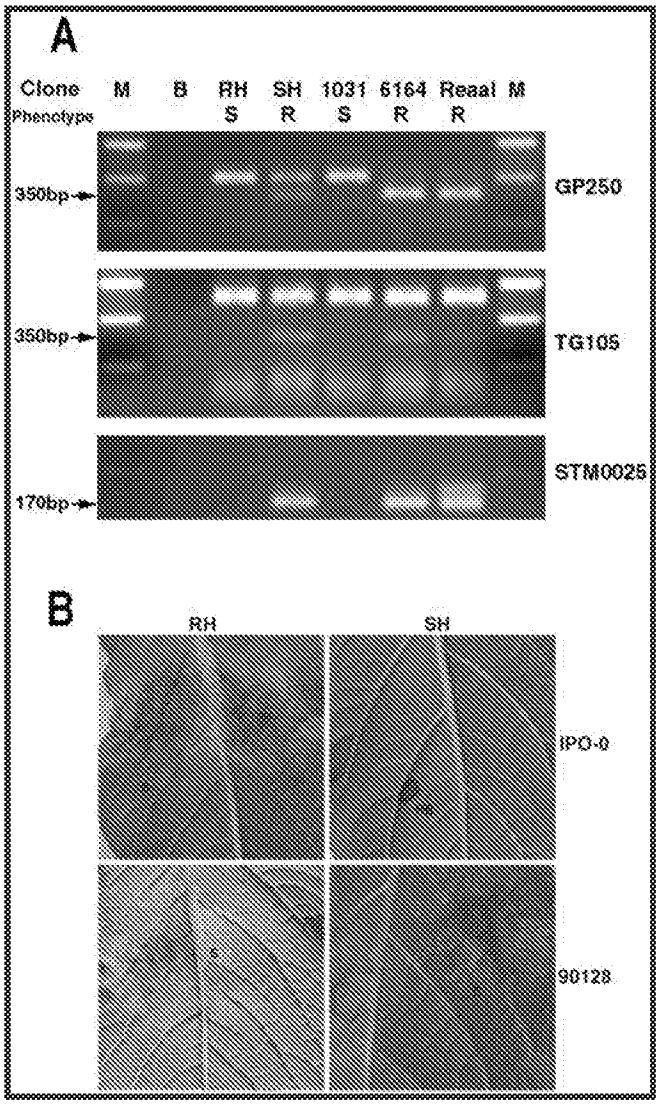


Fig. 1. Molecular and biological evidence for the presence of *R3* in SH83-92-488 (SH). **A,** Analysis of the genomic DNA from RH89-039-16 (RH), SH, 84-1031-29, J91-6164-21, and Reaal with markers GP250 (*Vsp*I), TG105 (*Hin*fI), and STM0025. The phenotype (R = resistant, S = susceptible) of these clones inoculated with *Phytophthora infestans* isolate IPO-0 is indicated. The sizes of polymorphic bands are indicated on the left. DNA size markers are indicated as 'M' and the blank control as 'B'. **B,** A gene-for-gene interaction of potato and *P. infestans*. RH and SH leaves inoculated with isolates IPO-0 (race 0) and 90128 (race 1.3.4.6.7.8.10.11), 5 days postinoculation. A race-specific resistance response occurred on SH leaves upon inoculation with IPO-0 (HR). Extensive sporulation (S) was noted in the other three compatible interactions.

Subsequently, the *R3* locus was mapped to the distal part of the short arm of chromosome 11 on the ultrahigh density AFLP map, confirming the previous map position in J91-6164 (El Kharbotly et al. 1994). Due to the limited population size (n = 130), the more than 10,000 AFLP markers cannot be localized individually. Instead of "centiMorgan" (cM), the genetic distance unit for the ultrahigh density map is "Bin". A Bin is defined as a genetic interval flanked by a recombination event on either side. All markers within a Bin are cosegregating. The length of a Bin is a genetic interval of approximately 0.8 cM (=$^1/_{130}$). *R3* was mapped in either Bin SH11B65 (65) or 66. Due to the loss of the diagnostic plant that could separate these two neighboring Bins, we can no longer precisely place *R3* to one of the two Bins. *R3* therefore cosegregates with all the markers mapped in the combined Bin 65/66, i.e., GP250, TG105, STM0025, and 43 cosegregating AFLPs. Bin 67 is the most distal Bin of the short arm of chromosome 11. Based on pedigree information, marker analysis, inoculation results, and genetic mapping, we conclude that SH carries *R3*.

Screening for informative recombination at the *R3* locus.

For high-resolution mapping of *R3*, it was essential to define the order of those cosegregating markers with meiotic crossovers. We applied a flanking marker strategy to screen for recombination events around the *R3* locus (Tanksley et al. 1995). Instead of laborious analysis with AFLP markers available from flanking Bins, we took advantage of the colinearity between tomato and potato at the *R3* region of chromosome 11 (Dong et al. 2000; Tanksley et al. 1992) and the sequences of known RFLP markers from the two species to develop easy-scoring PCR markers.

Based on their position, markers cLET5E4, TG26, and CT120 from tomato and St3.3.11, St1.1, and GP185 from potato were chosen for PCR marker development (Table 2). St3.3.11 was mapped in Bin 46, about 16 cM proximal to the *R3* locus. Like GP250 and TG105, cLET5E4, GP185, St1.1, and TG26 colocalized with *R3* in the ultrahigh density map and then were mapped in Bin 65/66. After testing with 250 additional SH × RH plants, we mapped the marker cLET5E4 proximal to the *R3* locus with three recombinants between the marker and the locus. CT120 was placed in Bin 67 and in repulsion to *R3*. It served as a distal flanking marker. Therefore, the cLET5E4-CT120 interval spans a chromosomal region with the *R3* locus in the center. We used these two markers for recombinant screening of 1,748 SH × RH progeny (Fig. 2). Out of these, we identified 34 recombinants between cLET5E4 and CT120.

Detection of two *R* genes at the *R3* locus.

The resistance phenotype of the 34 recombinants was determined with the *P. infestans* isolates 89148-9, H30P04, and IPO-0, which all have an incompatible interaction with SH. A

Table 3. *Phytophthora infestans* isolates used in this study and their virulence profiles before and after this study

Isolate	Virulence profile		Source
	Before	After	
89148-9	0	0	F. Govers, Wageningen University, The Netherlands
IPO-0	0	3b	W. Flier, Plant Research International, The Netherlands
H30P04	7	3a.7	F. Govers
89148-27	3.7	3a.3b.7	F. Govers
90128	1.3.4.6.7.8.10.11	1.3a.3b.4.6.7.8.10.11	F. Govers

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total of 26 recombinants were either resistant or susceptible to all three isolates. Surprisingly, we found the remaining eight recombinants differed in their response to H30P04 and IPO-0. Six of these, SW8536-18, SW8537-033, SW8539-004, SW8540-025, SW8540-054, and SW8540-309, were resistant to *P. infestans* isolate IPO-0 and susceptible to isolate H30P04, while the other two recombinants, SHRHC8-#130 and SW8540-325, were just the opposite, i.e.,, susceptible to isolate IPO-0 and resistant to isolate H30P04. All eight of these recombinants were resistant to 89148-9. The differential reaction to the isolates was confirmed with repeated inoculations using detached leaves and in vitro-grown plantlets (Fig. 3).

The recombinant SHRHC8-#130 is from the mapping population SH × RH of the ultrahigh density map, and it has a cross-over in the cLET5E4-CT120 interval. To exclude the possibility of a second locus that is not within the interval and affects the resistance to *P. infestans* isolate H30P04, we inoculated the mapping population SH × RH with H30P04 as we had previously done with isolate IPO-0. None of the 108 plants tested, but SHRHC08-#130 displayed a differential reaction to these two isolates. Genetic mapping revealed that resistance to isolate H30P04 in the population was inherited as a single *R* gene and was mapped within the cLET5E4-CT120 interval. No additional genetic component outside the interval affected the phenotype.

All the 34 recombinants in the cLET5E4-CT120 interval were subjected to characterization with all molecular markers from Bin 65/66 and Bin 67. The eight recombinants that showed a differential response to *P. infestans* isolates IPO-0 and H30P04 were found to carry a cross-over in the GP250-EATCMAGC_15 interval, which is a subinterval of the cLET5E4-CT120 interval (Fig. 4A and B). The remaining 26 recombinants that were either resistant or susceptible to both isolates did not have a cross-over in the subinterval. The marker pattern of the 34 recombinants indicated that the difference in isolate specificity was due to cross-overs between two closely linked *R* genes with distinct resistance spectra.

The data on isolate specificities, mapping, and marker pattern clearly demonstrated that the phenotype of *R3* resistance in SH is conferred by two closely linked *R* genes. Hereby, we designate them as *R3a* and *R3b*. *R3a* confers resistance to isolate IPO-0 and not to H30P04, and *R3b* vice versa. Both genes confer resistance to isolate 89148-9. Associated with the nomination of *R3a* and *R3b*, we can now designate IPO-0 as race 3b and H30P04 as race 3a.7. The isolate 89148-9 is still race 0 in this scenario (Table 3).

Construction of a high-resolution map at the *R3* locus.

The 34 recombinants identified from the cLET5E4-CT120 interval from the 1,748 SH × RH plants divided the interval into 35 sub-Bins (Fig. 4B). The genetic length of a sub-Bin is equivalent to approximately 0.05 cM (=$^{1}/_{1748}$). The resolution achieved with the high-resolution map of the *R3* locus is 16 times higher than that of the ultrahigh density map (0.05 versus 0.8 cM). A total of 35 molecular markers (27 AFLP markers and eight PCR markers) from the interval were assigned to the sub-Bins. The distribution of cross-overs and markers was nonrandom within the cLET5E4-CT120 interval. Nearly one third of the markers were clustered in sub-Bin 20, while no marker was placed in 22 of the 35 sub-Bins (Fig. 4B).

The *R3a* gene resides in sub-Bin 20, cosegregating with GP250, STM0025, and 10 AFLPs. TG105 and four AFLP markers were the closest proximal markers, separated from *R3a* by two recombination events. GP185, TG26, and EACAMCAG_152.6 were the closest distal markers, separated from *R3a* by three recombination events (Fig. 4B). The order of TG105-GP250-GP185 in SH was well consistent with the previous mapping result (Gebhardt et al. 1994). A total of 20 markers clustered in the TG105-GP185 interval that consists of six sub-Bins.

The *R3b* gene was mapped in sub-Bin 28, 8 sub-Bins (approximately 0.4 cM) distal to *R3a* (Fig. 4B). No marker cosegregates with the gene. The AFLP markers PCAMATA_4 and EATCMAGC_15 are its closest markers, separated by three and two recombinations, respectively. The PCAMATA_4-EATCMAGC_15 interval for *R3b* spans the same genetic distance as the TG105-GP185 interval of *R3a*, i.e., six sub-Bins, but contains much fewer markers (2 versus 20).

A natural recombination between *R3a* and *R3b* in *S. demissum*.

Introgression analysis based on pedigree information can provide additional resolution to determine the order of markers and genes of interest, and thus, it can serve as a supplemental tool to fine mapping based on segregating population (Kanyuka et al. 1999). Reaal is one of the best-characterized clones that carry the *R3* locus introgressed from *S. demissum* (Mastenbroek 1953). There are five generations of crossing and one round of dihaploidization between Reaal and SH (data not shown). The introgression study with markers tightly linked to *R3a* (GP250, STM0025, and TG105) between SH

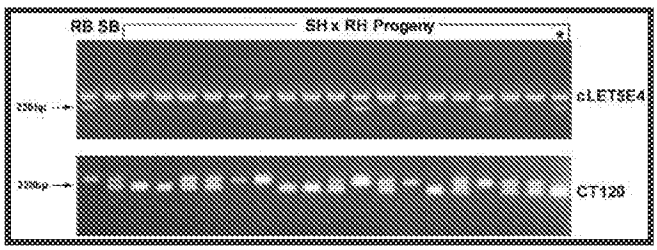


Fig. 2. Screening for recombination events near the *R3* locus in the SH83-92-488 × RH89-039-16 progeny with two flanking markers, cLET5E4 (*Hha*I) and CT120 (*Tsp*509I). The sizes of the diagnostic bands are indicated on the left. RB = resistant bulk consisting of 20 resistant plants; SB = susceptible bulk consisting of 20 susceptible plants. The presence of the 220-bp band of cLET5E4 is associated with the absence of the 320-bp band of CT120 in all progeny except in the recombinants. The right lane (*) shows a recombinant pattern.

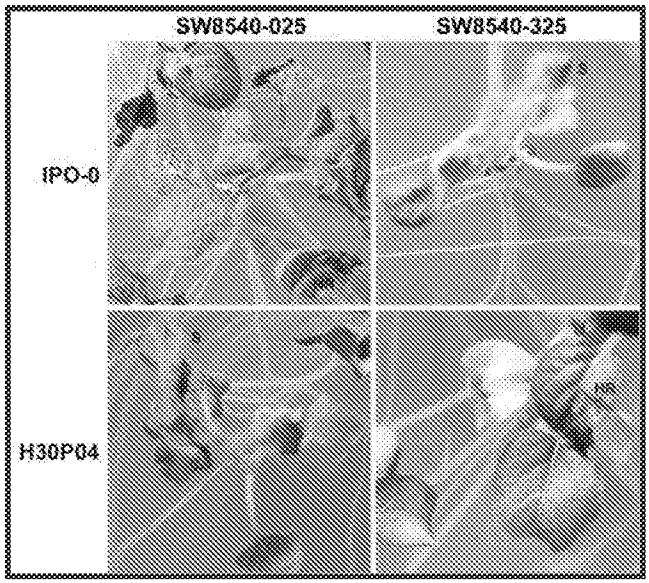


Fig. 3. Phenotype of differential resistance response at 6 days postinoculation with *Phytophthora infestans* isolates IPO-0 and H30P04 on in vitro plants. SW8540-025 is resistant (HR) to IPO-0 and is susceptible to H30P04 as shown by the sporulating lesions (S). SW8540-325 shows the opposite response.

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and Reaal suggested that *R3a* in SH descended from Reaal (Fig. 1A). However, these data cannot exclude the possibility that *R3b* in SH was derived from another clone rather than Reaal and *S. demissum*.

To check the origin of *R3b* and to confirm the SH-Reaal link on *R3a*, we conducted a fine comparison of SH, Reaal, two *S. demissum* accessions (17810 and 18313) (Table 1), and four susceptible *S. tuberosum* clones (1024, 1031, CE, and KA) (Table 1) with all 29 coupling phase markers in the cLET5E4-CT120 interval. SH, Reaal, and 17810 showed identical marker patterns in the interval (Fig. 4A and C). Notably, 17810 is synonymous to CPC2127, one of the R3 donor accessions of *S. demissum* (Black 1950). This similarity was not found for any of the four susceptible clones (1024, 1031, CE, and KA). Their marker pattern at the interval was almost identical to that of RH, the susceptible parental line. In the 29 coupling phase markers analyzed, only one marker pattern differed between 1031, CE, KA, and RH, and four differed between 1024 and

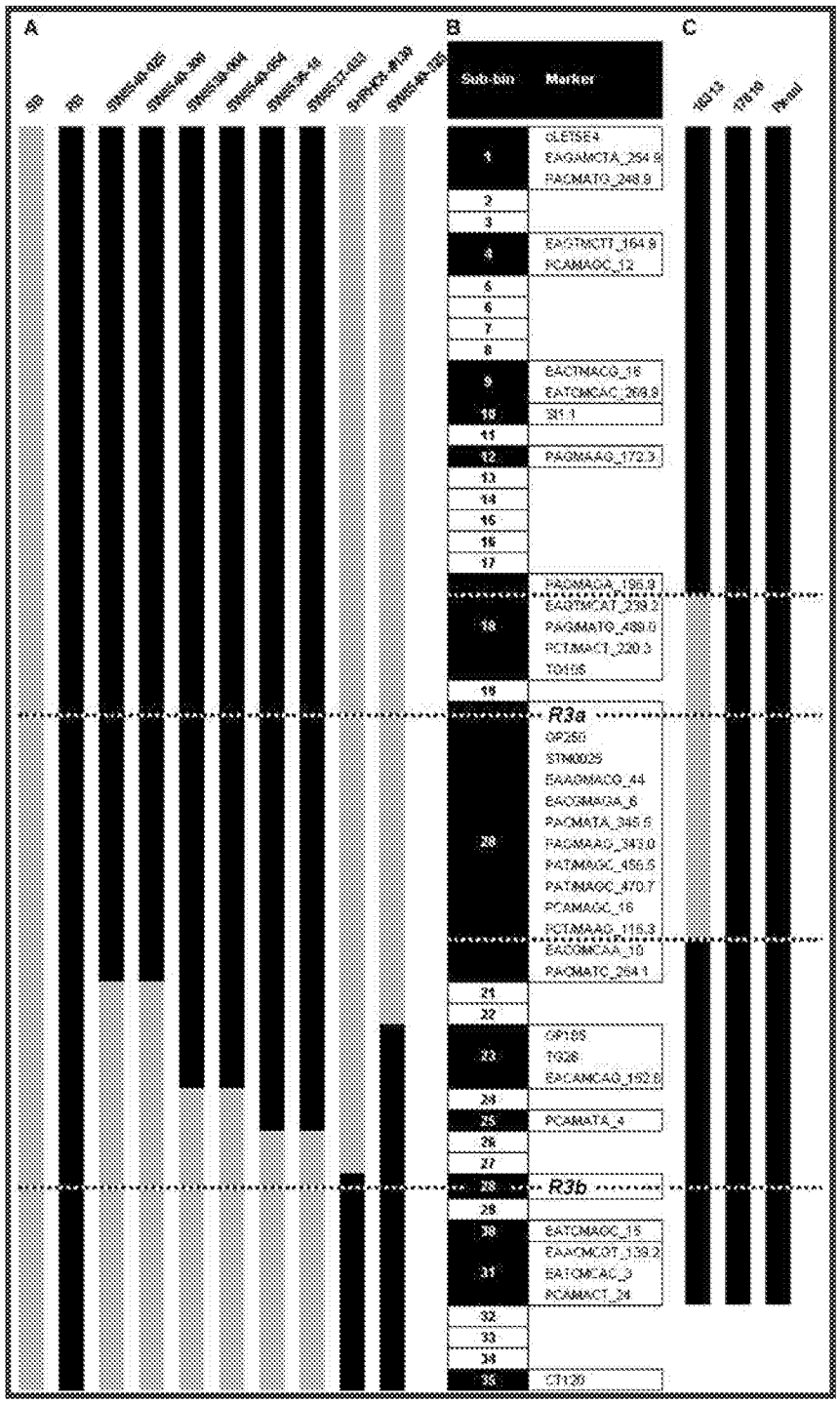


Fig. 4. High-resolution genetic map of *R3a* and *R3b*. **A,** Graphical genotypes of the susceptible bulk (SB, gray bar), the resistant bulk (RB, black bar), and the eight SH83-92-488 × RH89-039-16 progeny that displayed a differential response to H30P04 and IPO-0. The transition of black and gray indicates a cross-over. **B,** The high-resolution map of the *R3* locus. The sub-Bins are numbered from 1 to 35 in a proximal-distal order. The sub-Bins filled with one or more markers, genes, or both are shown with black background and white font. The dotted lines indicate the positions of *R3a* and *R3b* on the genotypes listed in A and C. **C,** Graphical genotypes of Reaal and two *S. demissum* accessions, 18313 and 17810. Reaal and 17810 are identical with RB. Two dotted lines, splitting sub-Bins 18 and 20 indicate the interval of a susceptible "island" at 18313. Marker CT120 was not investigated in the three clones, because it is a repulsion phase marker.

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RH. Together with pedigree information, this result strongly supports the notion that the *R3a-R3b*-carrying chromosome region in SH descended from Reaal and originated from *S. demissum*.

Interestingly, marker analysis revealed that another *S. demissum* accession, 18313, contains an "island" of susceptible alleles from sub-Bin 18 to 20, flanked by two blocks of resistance alleles (Fig. 4B and C). The clustering of markers in the island was confirmed by physical mapping (data not shown). The island in 18313 may offer a further resolution for determination of marker order relative to *R3a* because sub-Bin 20 can be divided into two parts based on the result. The AFLP markers EACGMCAA_10 and PACMATC_264.1 were separated from the other markers in sub-Bin 20 (Fig. 4B and C). From the marker pattern, 18313 was predicted to carry *R3b*.

The accession 18313 was subsequently tested for resistance specificity with *P. infestans* isolates 89148-9, IPO-0, and H30P04. It did display a differential reaction to these isolates that was the same as the reaction of the recombinants SHRHC8-#130 and SW8540-325, i.e., resistant to 89148-9 and H30P04 but susceptible to IPO-0. Therefore, we conclude that 18313 does carry *R3b* but not *R3a* and is a natural recombinant of these two *R* genes. The characterization of this accession not only confirmed the presence of the two functionally distinct genes at the *R3* locus but also further delimited *R3a* to the upper part of the otherwise intact sub-Bin 20 (Fig. 4B and C). Thus, the introgression analysis resulted in additional resolution instrumental for fine mapping the *R3* locus.

DISCUSSION

In this report, we describe the construction of a high-resolution genetic map reaching a 0.05-cM resolution at the *R3* locus and the finding that the locus consists of two distinctly functional *R* genes, *R3a* and *R3b*. The high resolution together with accurate disease testing with specific *P. infestans* isolates enabled the detection of two closely linked *R* genes with different resistance spectra. The introgression analysis confirmed that both genes in SH were derived from *S. demissum* and provided a further resolution to narrow down the interval for the *R3a* gene. The resolution obtained from both recombination analysis and introgression study will assist the isolation of both genes using a positional cloning strategy.

For high-resolution genetic mapping, two aspects are essential, marker saturation, i.e., the amount of sequence polymorphisms in the target region, and recombination frequency, i.e., the amount of meiotic cross-overs to resolve the markers. However, these two factors are often negatively correlated. Recombination in general is inhibited by sequence heterogeneity (Borts and Haber 1989; Dooner and Martinez-Ferez 1997; Metzenberg et al. 1991). The frequency of mitotic recombination in yeast can be affected noticeably by less than 1% nucleotide sequence divergence (Datta et al. 1997). Recombination frequency is about eightfold higher in the *Mi* region of a *Lycopersicon peruvianum* cross than a *L. esculentum* cross with *Mi* introgression from *L. peruvianum* (Kaloshian et al. 1998). This is also the case for the *R3* locus in SH, where we found a significant association between sequence heterogeneity, measured by the number of markers per cM and the reduction of recombination frequency using the tomato map as reference (Fulton et al. 2002; Tanksley et al. 1992). The cLET5E4-TG105, TG105-TG26, and TG26-CT120 intervals in SH have 16.5, 80, and 15 markers per cM, respectively. This is correlated to 9, 16, and 4 times less recombination, respectively (r = 0.918). *R3b* resides in a region with higher recombination frequency but less saturation than *R3a*.

Most of the *R* genes that have been characterized at the molecular level belong to complex loci with tightly linked paralogs. Large arrays of similar sequences allow for equal or unequal recombination events, resulting in the formation of new gene family members (Ellis et al. 2000; Hulbert et al. 2001; Michelmore and Meyers 1998). However, the evolutionary fate of new members in complex resistance loci may largely depend on the interaction between plants and their microbial environment. The majority of complex resistance loci carry a functional *R* gene and multiple paralogs with no detectable function, although they are not obvious pseudogenes and appear capable of coding for proteins similar to the functional *R* genes (Hulbert et al. 2001). Only a few complex resistance loci comprise more than one detectable functional *R* gene (Botella et al. 1998; Dixon et al. 1996; Parniske et al. 1997; Takken et al. 1999; van der Vossen et al. 2000; Wang et al. 1998). In contrast to the *I1* and *RB or Rpi-blb1* loci, where only one functional gene for *P. infestans* resistance was found in each locus (Ballvora et al. 2002; Song et al. 2003; van der Vossen et al. 2003), the *R3* locus harbors two *R* genes with distinct resistance specificities to the oomycete pathogen.

Map positions of resistance genes appear to be well-conserved in members of the family Solanaceae (Grube et al. 2000; Pan et al. 2000). The *R3* locus was mapped on the short arm of chromosome 11 in potato (syntenic to the long arm of chromosome 11 in tomato), where potato and tomato show a high macro-colinearity (Tanksley et al. 1992). Up to date, the *SL8* (*I2*) gene family in tomato was the only *R* gene family that has been molecularly characterized on this chromosome

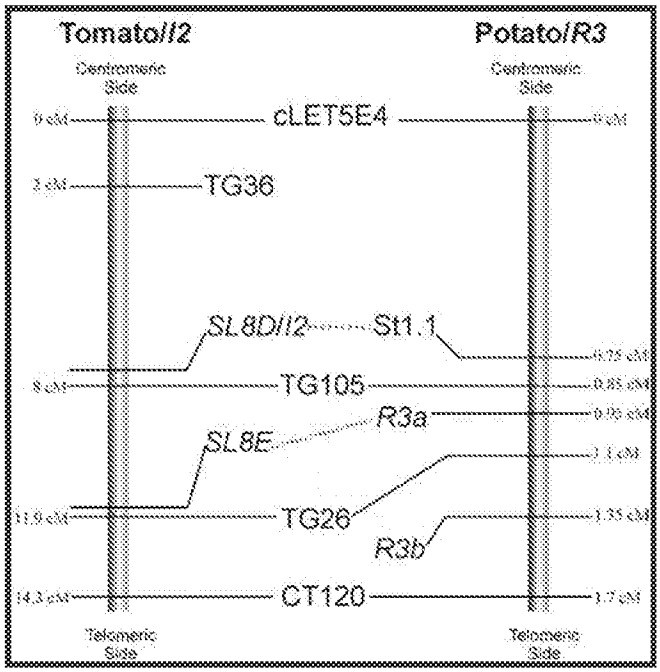


Fig 5. The synteny between the *R3* locus on the short arm of chromosome 11 in potato clone SH83-92-488 and the *I2* locus on the long arm of chromosome 11 of tomato. The left and right solid bars represent the tomato and potato chromosome, respectively. Mapped markers or genes are connected by black lines. On the tomato map, the positions of TG36, TG105, TG26, and CT120 were according to Tanksley and associates (1992), cLET5E4 according to Fulton and associates (2002) and to the Solanaceae Genomics Network, and *SL8D/I2* and *SL8E* according to Ori and associates (1997). The positions of markers and genes on the potato map were determined in this study. For the convenience of comparison, the 'sub-Bin' distances in potato have been changed into the 'cM' distances (1 sub-Bin ≅ 0.05 cM). The dotted line indicated the putative relation between *SL8D* and St1.1 and *SL8E* and *R3a*, respectively.

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arm. This gene family distributes to five genomic positions in tomato, and three of these, *SL8C*, *SL8D*, and *SL8E* (in order from the centromere to telomere), were mapped to this chromosome arm (Ori et al. 1997; Simons et al. 1998). The *SL8C* cluster maps within 10 cM of the *Stemphylium* spp. *R* gene *Sm* (Behare et al. 1991). The *SL8D* cluster lies between TG36 and TG105 and contains *I2* and six paralogs (Ori et al. 1997; Simons et al. 1998). The potato marker St1.1 has 91% nucleotide homology with the NBS region of *I2* and localizes between cLET5E4 and TG105. Therefore, St1.1 may be regarded as one of the potato orthologs of the *SL8D* locus (Fig. 5). In tomato, the *SL8E* locus resides between TG105 and TG26 (0.25 cM to TG26) and consists of only one paralog, as indicated by Southern blot analysis (Ori et al. 1997). In this study, *R3a* was also mapped in the TG105-TG26 interval, possibly resembling the *SL8E* locus in tomato. *R3b* is distal to TG26 (Fig. 4B and 5). Moreover, we found several *I2*GAs (tomato *I2* resistance gene analogs) cosegregating with *R3a* and *R3b* (data not shown), but the relationship of these *I2*GAs with *R3a* and *R3b* is yet to be determined. At the *SL8E*-syntenic region of *S. demissum*, *I2*GAs not only grew in number but also duplicated over TG26. It will be interesting to investigate whether this expansion is associated with the capacity to recognize additional elicitors from *P. infestans*.

The genomic region of *R3* is rich in functional diversity for *P. infestans* resistance. Besides *R3*, two additional haplotypes, *R6* and *R7*, confer distinct specificities (El-Kharbotly et al. 1996). The *R3* locus might have passed through multiple rounds of gene duplication and diversifying selection to produce new specificities for *P. infestans* resistance and, thus, could be a good example to study the dynamic evolution of the potato genome in the perspective of coevolution with this (in)famous oomycete pathogen.

MATERIALS AND METHODS

Plant materials.

Plant materials used in the study and their resistance profiles are listed in Table 1. The two *S. demissum* accessions were provided by the Center for Genetic Resources of the Netherlands (Wageningen). All other plant materials were from the Laboratory of Plant Breeding. The population segregating for *R3* was derived from a diploid cross between SH as maternal clone and RH as paternal one. From this cross, 130 progeny have been used for constructing the ultrahigh density map. For high-resolution mapping, 1,618 additional plants from the same cross were used. In total, 1,748 individuals were used to search for recombination events occurring near *R3*. For introgression study, five plants from each *S. demissum* accession were used for inoculation. From each accession, DNA pooled from the five individual plants was subjected to molecular marker analysis.

P. infestans isolates and disease test.

P. infestans isolates used in the study and their virulence profiles are listed in Table 3. Their virulence profiles were confirmed using a standard differential set of potato clones (van der Lee et al. 2001). Inoculum preparation was performed as described previously (Vleeshouwers et al. 1999). Resistance testing was performed on either detached leaves (Vleeshouwers et al. 1999), in vitro plantlets (Huang et al., *unpublished data*), or both. Resistance scoring was performed as described by van der Lee and associates (2001).

DNA isolation.

Genomic DNA from plants grown in greenhouse was isolated according to van der Beek and associates (1992). For DNA extraction of in vitro seedlings, the above method was modified to a high-throughput procedure, using the Retsch machine (Retsch Inc., Haan, Germany) and 96-deep-well Costar microtiter plates (Corning Inc., Corning, NY, U.S.A.).

Molecular markers.

PCR markers were derived from RFLP/SSR markers that have been previously mapped on chromosome 11 of potato (GP185, GP250, St1.1, St3.3.11, and STM0025) and tomato (cLET5E4, CT120, TG26, and TG105) (Gebhardt et al. 1994; Leister et al. 1996; Milbourne et al. 1998; Tanksley et al. 1992). An overview of the markers is given in Table 2. The sequence of GP185 was provided by B. Baker, University of California, Berkeley, CA, U.S.A. Other sequences were obtained from the NCBI database, from the Solanaceae Genomics Network, or from our own sequencing project. Primer pairs were designed with the PrimerSelect module of the DNASTAR package (DNASTAR Inc., Madison, WI, U.S.A.). For PCR analysis, 15-µl reaction mixtures were prepared, containing 20 ng of DNA, 7.5 ng of each primer, 0.1 mM of each dNTP, 0.3 units *Taq* polymerase (Supertaq, Enzyme Technologies Ltd, Cambridge), 10 mM Tris-HCl, pH 9, 1.5 mM $MgCl_2$, 50 mM KCl, 0.1% Triton X-100, and 0.01% (wt/vol) gelatin. The PCR were performed for 35 cycles, using the following profile: 30 s DNA denaturation at 94°C, 30 s annealing at melting temperature (Table 2), and 45 s elongation at 72°C. As a first step in PCR amplification, DNA was denatured for 5 min at 94°C and finalized by an extra 7-min elongation step at 72°C. The amplification reactions were performed in a Biometra T-gradient thermocycler (Westburg, Leusden, The Netherlands). Depending on the marker, the PCR product was digested with an appropriate restriction enzyme (Table 2). Subsequently, the (digested) PCR products were analyzed by electrophoresis in agarose gels.

AFLP analysis (Vos et al. 1995) was performed on a Licor sequencer (LI-COR, Lincoln, NB, U.S.A.), using fluorescent-labeled *Eco*RI or *Pst*I primers.

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AUTHOR-RECOMMENDED INTERNET RESOURCES

European Union project to develop an ultrahigh density (UHD) map of the potato: www.dpw.wageningen-ur.nl/uhd)

The Solanaceae Genomics Network: www.sgn.cornell.edu

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Comparative genomics enabled the isolation of the *R3a* late blight resistance gene in potato

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Summary

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. 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. In this report, we adopted a local RGA approach using genomic information from the model Solanaceous plant tomato to isolate *R3a*, a potato gene that confers race-specific resistance to the late blight pathogen *Phytophthora infestans*. *R3a* is a member of the *R3* complex locus on chromosome 11. Comparative analyses of the *R3* complex locus with the corresponding *I2* complex locus in tomato suggest that this is an ancient locus involved in plant innate immunity against oomycete and fungal pathogens. However, the *R3* complex locus has evolved after divergence from tomato and the locus has experienced a significant expansion in potato without disruption of the flanking colinearity. This expansion has resulted in an increase in the number of *R* genes and in functional diversification, which has probably been driven by the co-evolutionary history between *P. infestans* and its host potato. Constitutive expression was observed for the *R3a* gene, as well as some of its paralogues whose functions remain unknown.

Keywords: *R3a*, comparative genomics, potato, tomato, disease resistance, *Phytophthora infestans*.

Introduction

Comparative genomics investigates the similarity and differences in structure and function of genomes across taxa. Full genome sequences of Arabidopsis (Arabidopsis Genome Initiative, 2000) and rice (Goff *et al.*, 2002; Yu *et al.*, 2002) and several other ongoing sequencing projects will offer unprecedented resources to study the evolution of sequence and function of orthologous genes and to understand diversification and adaptation. A fundamental and practical question in comparative genomics is whether this vast amount of sequence information from model plant species will facilitate the cloning of genes with agronomic importance from crop species with larger genomes. Resistance to plant pathogens, often defined by single dominant disease resistance (*R*) genes (Dangl and Jones, 2001), is an important crop trait that could benefit from the sequencing of model species. This benefit, however, will largely depend on the given plant family. Extensive loss of colinearity has been reported within crucifers and grasses (Gale and Devos, 1998; Paterson *et al.*, 2000). Comparative analysis based on DNA sequences has revealed that disease resistance (*R*) loci may be evolving faster than the rest of the crucifer and grass genomes (Gale and Devos, 1998; Leister *et al.*, 1998; Paterson *et al.*, 2000). In contrast, the remarkable conservation of gene order (Bonierbale *et al.*, 1988) makes the Solanaceae, a botanical family that includes many important crops such as tomato and potato, an attractive subject for comparative genomics. Low-resolution comparative mapping indicated that *R* loci may be positionally conserved within Solanaceae (Grube *et al.*, 2000; Leister

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et al., 1996; Pan *et al.*, 2000). To date, this knowledge has never led to the isolation of a new *R* gene with known function.

The distal end of the long arm of chromosome 11 of tomato is a particularly interesting genomic region to investigate the evolution of *R* loci in a comparative genomics perspective. First, the overall structural colinearity is excellent between tomato and potato (Tanksley *et al.*, 1992) and relatively good between tomato/potato and pepper (Livingstone *et al.*, 1999). Secondly, this genomic region is a hotspot for *R* genes, harboring major genes encoding resistance to the fungi *Fusarium oxysporum* (*I2*, Ori *et al.*, 1997; Simons *et al.*, 1998) and *Stemphylium* spp. (*Sm*, Behare *et al.*, 1991), and to yellow leaf curl virus (Hanson *et al.*, 2000) in tomato, to the oomycete *Phytophthora infestans* (*R3*, *R6*, and *R7*, El Kharbotly *et al.*, 1994, 1996) in potato, and to tobacco mosaic virus (*L*, Lefebvre *et al.*, 1995) in pepper. The region also contains several quantitative trait loci conferring resistance to the cyst nematode *Globodera rostochiensis* (*Gro1.3*, Kreike *et al.*, 1993), to *P. infestans* (*phyt7*, Oberhagemann *et al.*, 1999) in potato, and to cucumber mosaic virus (*cmv4*, Grube *et al.*, 2000) and to *P. capsici* (*phyt 3*, Lefebvre and Palloix, 1996) in pepper. Last, the molecularly well characterized *I2* complex locus in tomato (Ori *et al.*, 1997; Simons *et al.*, 1998) provides an excellent template for a comparative study. The complex locus consists of two clusters, *SL8D* and *SL8E*. The *SL8D* cluster contains seven coiled-coil nucleotide binding site and leucine-rich repeat (CC-NBS-LRR) type *R* gene sequences, including the *I2* gene conferring complete resistance to race 2 of *F. oxysporum* f. sp. *lycopersici* (Simons *et al.*, 1998) and the *I2C-1* (Ori *et al.*, 1997) and *I2C-5* (Sela-Buurlage *et al.*, 2001) genes conferring partial resistance to the same pathogen.

Potato is the most important non-cereal crop plant. One of the major constraints to potato production in the world is the late blight disease caused by the oomycete *P. infestans* (Fry and Goodwin, 1997). There is considerable interest in identifying late blight *R* genes and in understanding their evolution and mode of action (Ballvora *et al.*, 2002; Song *et al.*, 2003; van der Vossen *et al.*, 2003). In a previous study (Huang *et al.*, 2004), we discovered that the *R3* complex locus at chromosome 11 of potato comprises two functionally distinct late blight *R* genes, *R3a* and *R3b*. Here, we use genomic information from the model Solanaceous plant tomato to clone *R3a*, a gene that confers race-specific resistance to *P. infestans*.

Results

The potato R3 *and tomato* I2 *regions are highly colinear*

We previously showed that the genomic regions harboring the *R3* late blight resistance locus in potato and the *I2* *Fusarium* wilt resistance locus in tomato are colinear (Huang *et al.*, 2004). To determine the extent of colinearity between these regions, we mapped seven tomato markers on the high-resolution genetic map of the *R3* complex locus (Figure 1). All seven markers retained their order in tomato and potato. We identified a cluster of *I2* gene analogues (*I2*GAs) in potato that was mapped 0.1 cM centromeric to the TG105 marker. This potato *I2*GA cluster positionally corresponds to the *SL8D* cluster of the *I2* complex locus in tomato and was therefore named the *St-I2* cluster. The perfect micro-colinearity within the TG105-cLET24J2A interval indicates that the *R3a* cluster in potato is syntenic to the *SL8E* cluster in tomato. Despite the fact that the counterpart of the *R3b* cluster was not detected in tomato, the orthologous relationships of *SL8D* versus *St-I2* and *SL8E* versus *R3a* point to an ancient *R* locus prior to the tomato–potato divergence.

R3a *candidates were identified using a local resistance gene analogue (RGA) approach*

Although the potato *R3a*-tomato *SL8E* synteny was established (Figure 1), we could not directly use it for *R3a* cloning as there was no sequence available from the *SL8E* cluster.

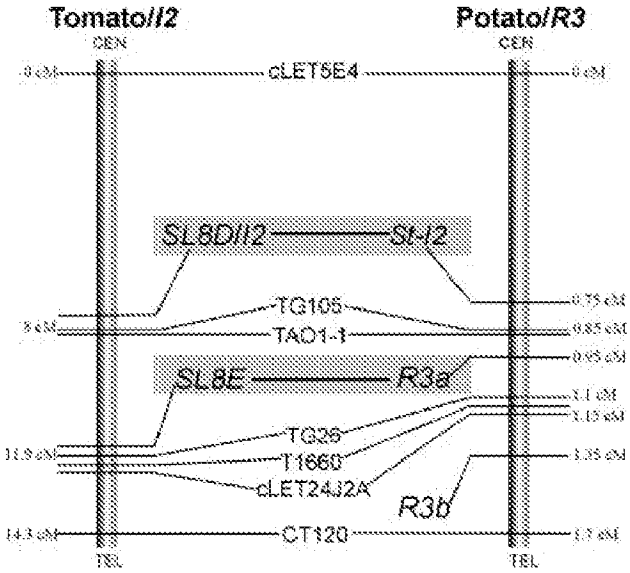


Figure 1. Comparative genetic maps of the *I2* complex locus in tomato and the *R3* complex locus in potato. The left and right solid bars represent the tomato and potato chromosomes, respectively. Orientation is indicated by CEN (centromere) and TEL (telomere). Mapped markers are connected by black lines. The syntenic relationships of *R* gene clusters are highlighted using gray rectangles. On the tomato map, the positions of TG105, TG26, and CT120 are according to Tanksley *et al.* (1992), cLET5E4, T1660, and cLET24J2A according to the Solanaceae Genomics Network (http://www.sgn.cornell.edu), and *SL8D* (containing the *I2* gene), TAO1-1, and *SL8E* according to Ori *et al.* (1997). On the potato map, the positions of cLET5E4, TG105, *R3a*, TG26, *R3b*, and CT120 were determined by a previous work (Huang *et al.*, 2004), TAO1-1, T1660, and cLET24J2A by aligning the marker sequences to the sequences of BACs mapped on the high-resolution map of *R3* (data not shown). The position of *St-I2* was determined as described in the text.

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However, the *SL8E* cluster was defined by cross-hybridization with the LRR part of the *I2C-1* gene from the *SL8D* cluster (Ori *et al.*, 1997). Using this information, a local RGA approach was applied. Instead of targeting conserved motifs within the NBS applied by global RGA approaches (Aarts *et al.*, 1998; Kanazin *et al.*, 1996; Leister *et al.*, 1996; van der Linden *et al.*, 2004; Yu *et al.*, 1996), the conserved sequences within the LRR of the *SL8D* cluster were used to design the P_{SL8D} primers (Table 1C). A BLAST search (Altschul *et al.*, 1990) for short, nearly exact matches proved the specificity of the P_{SL8D} primers, which hit no sequence other than the *SL8D* cluster with a reasonable low *E*-value. On DNA from the parental clones SH83-92-488 (SH) and RH89-039-16 (RH), the primers amplified a major smearing band of approximately 1 kb (data not shown), indicating that many copies of *I2*GAs with similar sizes were amplified. The size fitted the prediction from the sequences of the *SL8D* cluster (884–1049 bp). The above findings confirmed the suitability of the P_{SL8D} primers for identifying *I2*GAs in potato.

To identify *I2*GAs physically close to *R3a*, an association analysis on bacterial artificial chromosome (BAC) pools was conducted. Similar to other physical mapping methods like radiation hybrid mapping (Cox *et al.*, 1990) or HAPPY mapping (Dear and Cook, 1993), our method is based on the frequency that a given marker and *I2*GAs co-appear in BAC pools to estimate physical distance between them. The mapping panel is represented by BAC pools that contain 384 BACs, equivalent to 0.05 genome.

To determine whether any *R3a* marker (Figure 2a) was physically close to an *I2*GA, we screened 255 BAC pools with

Table 1 Molecular markers and primers used, their sequence, annealing temperature (T_m), and restriction enzyme used to generate polymorphism

	Marker	Primer (5′–3′)	T_m (°C)	Restriction enzyme
A	cLET5E4	CCA GGC ATG CTC AAT TTG GAG T TTC CCT GTT TGG ACT ACT TGT GGA	55	*Hha*I
	CT120	CGA GGG GGC GAA GGA TT CCA TGA GAT AAA CGA GGA ACC AGT	52	*Tsp*509I
B	241S	GGC ACT GAT AAG TTT TGG TTT TG GTG GTT TAT GAA TTG AAC TCA TGC	55	a.s.[c]
	194T	AAG CTT GAA TTC GTG GAC GG CTT TAT ACC AAC AGG TTG CTC	55	a.s.
	4T	AAG CTT TCA AAC CAA AAT GC AAA ATG ACT TTA CGT GGT CT	52	a.s.
	GP250	ACC AGT AGG ACC ACC ACC AAC AAT GAT CGT GAC GGC TCT ACT CTT TTA TGA	60–52[b]	*Vsp*I
	GP250-2	CTG GTA ATA GTA GTA ATG ATT CTT CGT C GAT CCT GAC TGC TCT ACT CTC TTA CGA	60–52[b]	*Vsp*I
C	P_{SL8D}	AAT TGG AGA GTT CCC TAC ACT TGA G AGG GAG GAG GGC AGT GCT GAT TC	50	*Alu*I
D	3RACE4	CGA AAG GAG TGG CAT TTA CAG AGA CGA	68–60[b]	
	3RACE5	CTT CCT CTA TTC AAA GGC TTA CCA TAG TG	68–60	
	3RACE6	CTC ACC TCT CTT CAA TAT CTA TTT ATT AGG	68–60	
	5RACE0	CAT TGT AAA CCG CTT TAG CAA GTG TTG TC	68–60	
	5RACE4	CAT TCC AAC AAT AGG AAC TAC AGT CCG C	68–60	
	5RACE5	CAT TCT GCC TTC CAA AGA TAT CAG AG	68–60	
	5RACE6	CAT CAC TTA GCA CAA TCT GAA GAC CG	68–60	
E	RT-SH23-1	GCT TCC GAT ATG TAT TGA TCT CAC G TGT GGC AAT CCT CTA CAA ACA ATG T	68–55[b]	
	RT-R3a	ATC GTT GTC ATG CTA TGA GAT TGT T CTT CAA GGT AGT GGG CAG TAT GCT T	68–55	
	RT-SH23-3	CGA CAT GTA ATT ATT TCA TGC CTC C AGA GGA ATT TCA AAC AAG GGA GTT C	68–55	
	RT-SH194-2	TGG ATT GAA AAG TTG CCT GAT TCT G CAA GGT AGT GGG CAG TAT GCT AAA T	68–55	
	Actin[a]	CAG CAA CTG GGA TGA TAT GG ATT TCG CTT TCA GCA GTG GT	68	

A, flanking markers; B, physical mapping; C, RGA marker; D, RACE; E, RT-PCR.
[a]Primers kindly provided by Chengwei Li of the Laboratory of Plant Breeding, Wageningen University.
[b]Touchdown PCR, −1°C/cycle.
[c]Allele-specific.

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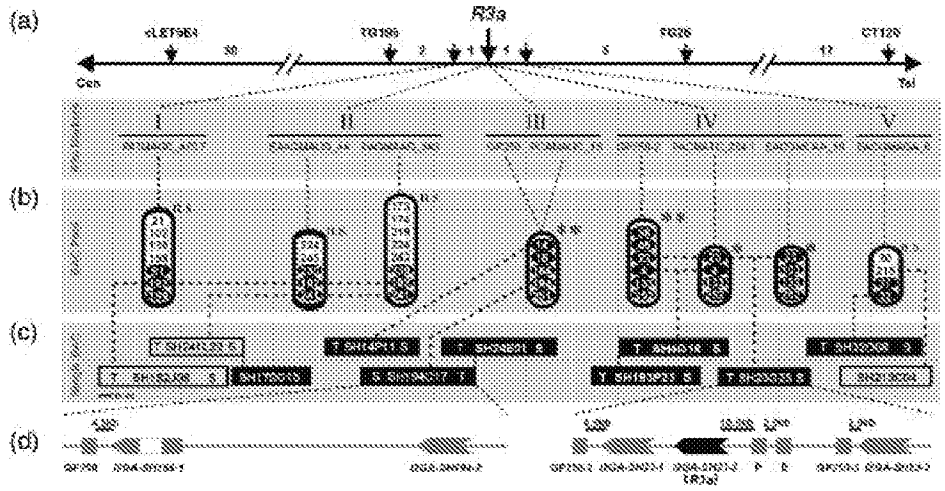


Figure 2. Genetic and physical mapping of *R3a*.
(a) Genetic map of *R3a*. Vertical arrows indicate the relative positions of markers linked to resistance. Numbers between the markers above the horizontal line indicate the number of recombinants identified in the 1748 SH × RH progeny used in a previous study (Huang *et al.*, 2004) and the additional 1200 progeny used in this study. The *R3a* markers are divided into five groups (I–V) (described in the text). GP250-2 was found in BAC pool screening, which is a copy of GP250 and also cosegregates with *R3a*.
(b) Physical association of markers and *I2*GAs. Oval beads within round-end rectangles represent set of BAC pools identified by a given marker in groups I–V. BAC pools with *I2*GA(s) are represented by a black background. The extent of association (n.s., not significant, $*P < 0.05$, $**P < 0.01$) between a given *R3a* marker and *I2*GAs on BAC pools are indicated on the upright side of each round-end rectangle.
(c) BAC Contigs at the *R3a* region. Rectangles present BACs and those carrying *I2*GA(s) are highlighted using black background. T (T7) and S (Sp6) indicate the orientation of the BACs. BACs SH182J06 and SH241L22 were identified by group I/II markers, SH14P11 and SH194C17 by group III markers, SH4G16 and SH23G23 by group IV markers, and SH32O08 and SH215C04 by group V marker. SH170O15, SH24E01, and SH193P23 were identified using BAC end markers 241S, 194T, and 4T (Table 1B), respectively.
(d) Location and orientation of I2GAs in SH194C17 and SH23G23 are shown. Single continuous ORFs were identified in *I2GA-SH194-2*, *-SH23-1*, *-2*, and *-3*. I2GA-SH194-1 is inserted by a retroelement (indicated by the white block). The positions of the markers GP250 and GP250-2 were identified by alignment. GP250-3 was found as another copy of GP250. The positions of the group IV AFLP markers EACGMCAA_10 (E), and PACMATC_264.1 (P) were defined *in silico* by identifying their restriction sites, selective nucleotides, and mobilities. The distance between markers and I2GAs is indicated above the annotated BACs.

the nine *R3a* markers and the P_{SL8D} primers. A total of 30 BAC pools were determined to be positive for one or more of the *R3a* markers, placing these markers in five groups on the basis of recombination events and co-occurrence in BAC pools (Figure 2a,b). The P_{SL8D} primers identified 92 *I2*GA-containing BAC pools, 19 of which coincided with those positive for the *R3a* markers. Chi-square tests showed that *I2*GAs were significantly associated with the markers in groups III and IV that cosegregated with *R3a* (Figure 2b). This suggested a close physical relationship between *R3a* and specific *I2*GAs, which therefore were regarded as *R3a* candidates.

From the BAC pools identified by the *R3a* markers and by chromosome walking, we isolated 11 BAC clones that span at least 700 kb (Figure 2c). Subsequently, BAC clone SH194C17 and SH23G23 were chosen for sequencing, as they were respectively identified by group III and IV markers (Figure 2c) and Southern hybridization (data not shown) indicated they contained five *I2*GAs. Annotation of the BAC sequences indeed predicted five *I2*GAs that were designated *I2GA-SH194-1*, *-2*, *I2GA-SH23-1*, *-2*, and *-3* (Figure 2d). Single continuous open reading frames (ORF) were present in *I2GA-SH194-2* and the three *I2*GAs in BAC SH23G23. *I2GA-SH194-1* is a pseudogene due to insertion of a Ty1/Copia-type retroelement. In the BAC sequences, the GP250 marker and the amplified fragment length polymorphism (AFLP) markers could also be recognized. The physical proximity between the *I2*GAs and the group III/IV markers (Figure 2d) validated the predicted association at the level of BAC pools (Figure 2b). The four *I2*GAs with full ORFs were considered as *R3a* candidates.

R3a *is an* I2GA

The genes *I2GA-SH194-1* and *-SH23-1*, *-2*, *-3* together with 2–3 kb up- and down-stream sequences were introduced into the susceptible clone 1029-31 and cv. Desiree via *Agrobacterium*-mediated transformation. At least 10 *in vitro* plantlets of each primary transformant were tested for resistance to each of the three *P. infestans* isolates 89148-9, IPO-0, and H30P04 in duplicate experiments. Cv. Desiree, 1029-31, RH, SH, and SW8540-025 (*R3a* recombinant) were included as controls. Only a 10.4-kb subclone containing *I2GA-SH23-2* was able to specifically complement the susceptible phenotype. All 15 primary transformants of this subclone exhibited *R3a*-specific resistance, that is, were resistant to isolate 89148-9 and IPO-0 and susceptible to H30P04 (Table 2 and Figure 3). The results were confirmed using a detached-leaf assay in duplicate tests. As the subclone contained only one ORF, we designated *I2GA-SH23-2* as *R3a*.

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Table 2 Disease test of primary transformants of *R3a* and its paralogues. Numbers of plants showing resistance to the three *Phytophthora infestans* isolate IPO-0, H30P04, and 89148-09 are indicated

Construct	Clone	No. plants tested[a]	No. plants showing resistance to *P. infestans* isolate[b]		
			89148-09	IPO-0	H30P04
*I2*GA-SH23-1	1029-31	12	0	0	0
	Desiree	6	0	0	0
*I2*GA-SH23-2 (*R3a*)	1029-31	8	8	8	0
	Desiree	7	7	7	0
*I2*GA-SH23-3	1029-31	13	0	0	0
	Desiree	5	0	0	0
*I2*GA-SH194-2	1029-31	23	0	0	0
	Desiree	8	0	0	0
Control	1029-31	10	0	0	0
	Desiree	10	0	0	0
	RH	10	0	0	0
	SW8540-025	10	10	10	0
	SH	10	10	10	10

[a]For constructs – number of independent transformants; for control – number of *in vitro* plantlets of the same genotype inoculated with each isolate.
[b]89148-09 and H30P04 kindly provided by Dr F. Govers of Laboratory of Phytopathology, Wageningen University and IPO-0 by Dr W. Flier of Plant Research International, Wageningen, The Netherlands.

The transcript structure of the *R3a* gene was determined by comparing the genomic sequence with cDNA fragments generated by random amplification of cDNA ends (RACE). The *R3a* transcript is 4176 nt long and encodes a predicted polypeptide of 1282 amino acids (a.a.) with a relative molecular mass of 145.9 kDa. The *R3a* gene and the *I2* gene (Simons *et al.*, 1998) have a similar intron–exon pattern and both have no intron in the coding region, but the intron close to the stop codon is much longer in *R3a* than in *I2* (Figure 4a).

The *R3a* gene encodes a putative CC-NBS-LRR protein and shares 88% DNA identity and 83% a.a. similarity to *I2* (Figure 4b). Considerable dissimilarity occurs at the CC domain, including a seven-a.a. indel. R3a and I2 proteins are quite conserved in the NBS domain (86% a.a. identity), especially at the motifs that define the domain (94% a.a. identity). The proteins mainly diverge at the LRR domain where two major differences were observed. First, although both proteins carry 29 LRR units, the R3a protein lacks a complete LRR unit of 28 a.a. at LRR 14/15 and contains an extra copy of a unique LRR unit of 23 a.a. at the LRR 26/27 (Figure 4b). Secondly, 45% (63/140) of the putative solvent-exposed residues (x in the frame xxLxLxx) differ between the I2 and R3a proteins.

The R3a and I2 proteins are more related to each other than to other known R proteins, as indicated by the zero *E*-value using BLASTP (Altschul *et al.*, 1990). Thus the *I2* and *R3a* genes belong to the same *R* gene family. Outside of this family, the most related known *R* gene is *Rpg1-b* ($E = e^{-176}$, 35% a.a. identity) from soybean mediating recognition of the Type III effector protein AvrB from *Pseudomonas syringae* (Ashfield *et al.*, 2004). The R3a protein bears only limited similarity (15 and 30% a.a. identity) to the other two known late blight R proteins, R1 (Ballvora *et al.*, 2002) and RB/Rpi-blb1 (Song *et al.*, 2003; van der Vossen *et al.*, 2003), respectively.

Dynamic evolution occurred in the R3a *cluster*

Despite the excellent overall colinearity in the *I2* and *R3* genomic regions (Figure 1), the *R3* region is physically larger in potato. In tomato the TG105-TG26 interval spans about 500 kb and contains one or few *I2*GAs, as demonstrated by long-range physical mapping (Simons *et al.*, 1998) and Southern analysis (Ori *et al.*, 1997). In contrast, the TG105-TG26 interval in SH spans more than 1 MB and contains at least nine *I2*GAs. The three contigs (11 BACs in total) in the *R3a* region (Figure 2c) span at least 700 kb. In addition, gaps between the depicted contigs and those between the depicted contigs and contigs harboring the markers TG105 and TG26 are predicted to be larger than 100 kb, the average

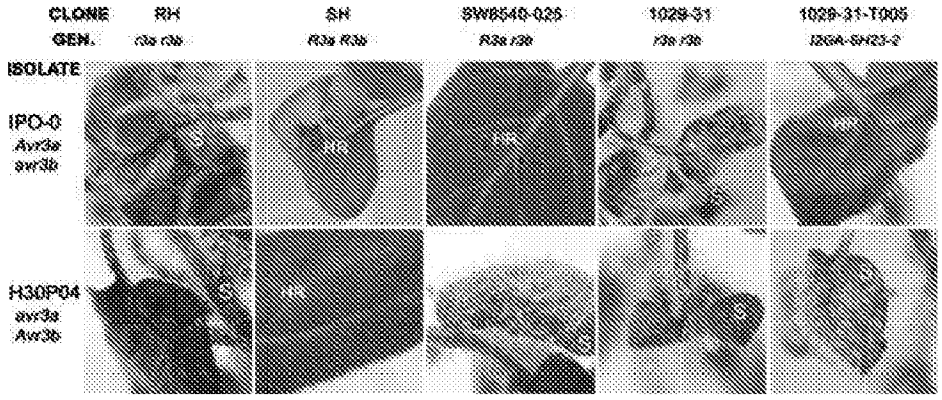


Figure 3. *In vitro* inoculation of the primary transformants of *R3a*. Massive sporulation (S) and localized hypersensitive reactions (HR) are observed on compatible and incompatible interactions, respectively. RH and 1029-31 are susceptible to both isolates. SH is resistant to both isolates. SW8540-025 (an SH × RH progeny carrying only *R3a*) and 1029-31-T005 (a 1029-31 transformant with *I2GA-SH23-2*) are resistant to IPO-0 (with *Avr3a*) and susceptible to H30P04 (without *Avr3a*).

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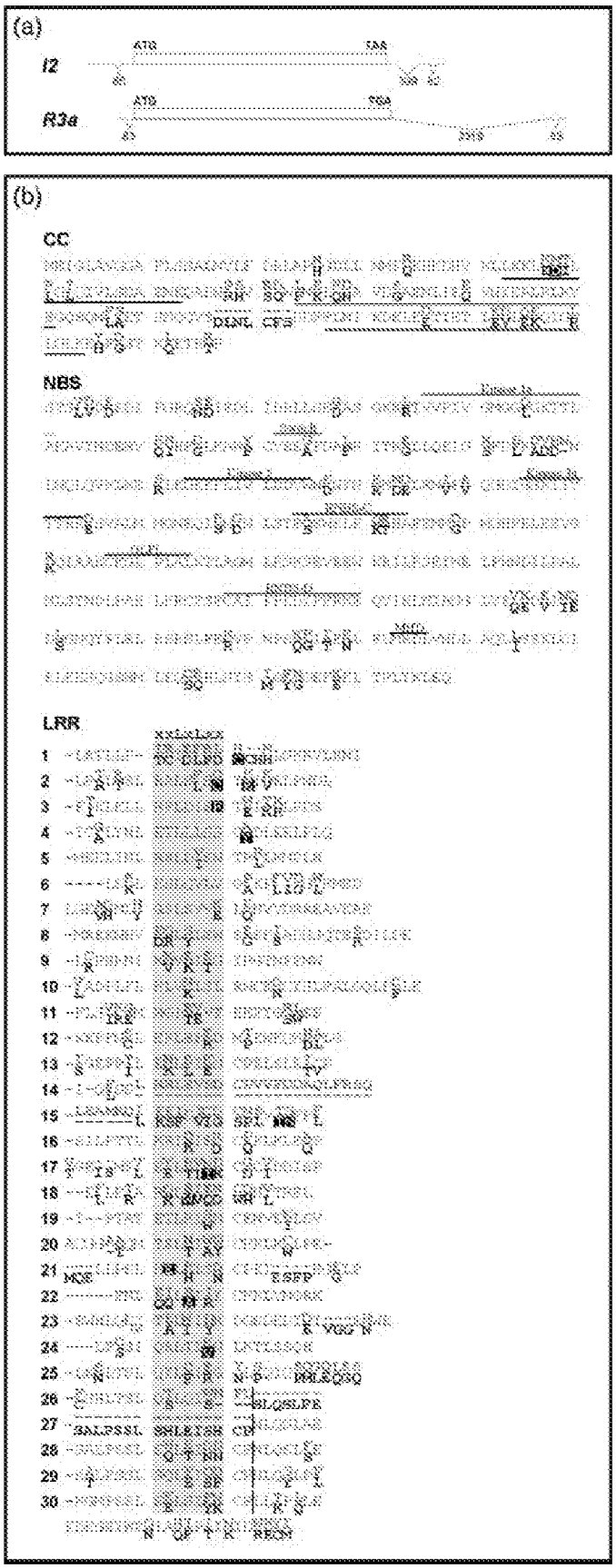


Figure 4. Comparison between *R3a* and *I2*.
(a) Schematic diagram of the transcript structures of *I2* and *R3a*. Horizontal lines indicate exons. Open rectangles represent coding sequences. Lines angled downwards indicate the position of introns, whose sizes are indicated below.
(b) Primary structure and alignment of the I2 and R3a resistance proteins. Amino acid residues of I2 and R3a that are identical are shown in normal script. I2- and R3a-specific residues are shown in bold at top and bottom lines, respectively. Dashes indicate deletion. The predicted coiled coils (Lupas *et al.*, 1991) in the CC domains are underlined. The conserved motifs of the NBS domain are overlined. The 30 LRR units in the alignment are numbered. The β-sheet (consensus xxLxLxx) of each LRR is highlighted with a gray background. The R3a residues under significant diversifying selection are highlighted with a black background. The calculation is based on the alignment of *R3a*, *I2GA-SH23-1*, *-3*, and *I2GA-SH194-2* using PAML (Yang *et al.*, 2000). Between vertical lines are the unique LRR units (consensus NLQSLAESALPSSLSHLEIDDCP) that display distinct copy numbers between *I2*, *R3a*, and their paralogues.

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insert size of the SH BAC library. In addition to the five *I2*GAs in BAC clone SH194C17 and SH23G23, we identified a new *I2*GA in each of the four BACs (SH170O15, SH24E01, SH193P23, and SH32O08, Figure 2c) by Southern blotting (data not shown). Altogether, these data indicate that the *R3* complex locus in SH has expanded significantly compared to that of tomato (cv. Motelle and Mogeor, Ori *et al.*, 1997; breeding line E22, Simons *et al.*, 1998), resulting in an increase in the number of *R* gene sequences. It remains unknown whether such variation in size and *R* gene copy number reflects *de facto* tomato and potato genomes or whether such variation also exists within each species.

Diversifying selection has been detected in many *R* gene complex loci (Hulbert *et al.*, 2001). Sites under diversifying selection were investigated using the program PAML (Yang, 1997; Yang *et al.*, 2000). Models M7 and M8 in the program 'codeml' of PAML were run for the four *I2*GAs (*R3a*, *I2GA-SH23-1*, *-3*, and *I2GA-SH194-2*) at the *R3a* cluster in potato. Model M7 is a special case of model M8 and assumes no selection, whereas model M8 allows for positively selected sites (Yang *et al.*, 2000). Diversifying selection can be confirmed using a likelihood-ratio test by comparing the likelihood calculated using models M8 and M7 (Yang *et al.*, 2000). Comparison of the results from model 7 and model 8 showed that the I2GAs at the *R3a* cluster have undergone diversifying selection ($\chi^2 = 92.8$, df = 2; $P < 0.001$). Selection at each site of the *R3a* homologues was calculated using model M8. Thirteen sites were found to be under significant diversifying selection (Figure 4b and Figure S1). Twelve of them are in the LRR domain and seven are putative solvent-exposed residues. This observation is compatible with the idea that the LRR region of an R protein mainly defines recognition specificities (Parniske *et al.*, 1997; Van der Hoorn *et al.*, 2001; Wulff *et al.*, 2001).

Putative sequence exchange between paralogues was previously observed at several *R* gene complex loci (Noel *et al.*, 1999; Parniske *et al.*, 1997) and is likely a mechanism for creating new specificities (reviewed in Hulbert *et al.*, 2001). Sequence relationships within the *R3a* cluster were analyzed by determining informative polymorphic sites (IPS, Parniske *et al.*, 1997). In total, 129 IPS were detected (Figure S2). The *R3a* and *I2GA-SH23-1* genes have 94% DNA identity and they share 91 IPS, suggesting that they are derived from the same ancestral gene. Interestingly, in the central part of the alignment, the *R3a* cluster exhibits a complex patchwork. The *R3a* and *I2GA-SH194-2* genes share sequence affiliations at three patches, comprising 21 IPS, which result in 10 non-synonymous substitutions between *R3a* and *I2GA-SH23-1*. An almost continuous sequence affiliation (nine IPS) between the *R3a* and *I2GA-SH23-2* genes was also found, comprising three non-synonymous substitutions. It remains unclear whether the putative sequence exchanges between the paralogues have lead to new resistance specificities in the *R3a* cluster.

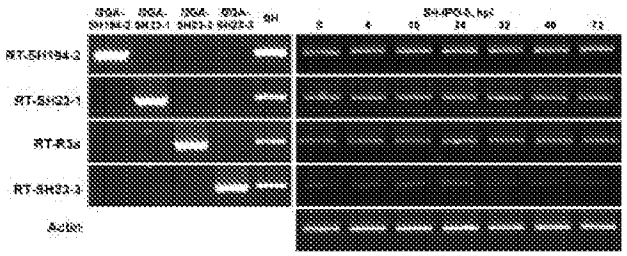


Figure 5. RT-PCR of the *R3a* cluster. Gene-specific primers used are listed on the left. Subclones with individual *I2*GAs (*I2GA-SH194-2*, *-SH23-1*, *-2*, *-3*) and genomic DNA of SH are used as control for the specificities of the primers. mRNA isolated from the detached leaves of SH inoculated with *Phytophthora infestans* isolate IPO-0 were used to study the expression pattern in a time course from 0 to 72 hpi. Actin primers were used to monitor mRNA quantity.

R3a *is constitutively expressed*

To examine the expression patterns of the genes *R3a*, *I2GA-SH23-1*, *-3*, and *I2GA-SH194-2*, gene-specific semiquantitative RT-PCR was performed on mRNA isolated with from *P. infestans* challenged (isolate IPO-0 and 90128) or mock-inoculated leaves of SH at 0, 8, 16, 24, 32, 48, and 72 h post-inoculation. All the four *I2*GAs are constitutively expressed in all treatments, but the expression level of *I2GA-SH23-3* is significantly lower than the other paralogues (Figure 5).

Discussion

In this study, we used genomic information from tomato to isolate the potato late blight resistance gene *R3a* from an ancient locus involved in plant innate immunity in the Solanaceae. Despite technological advances in genomics in recent years, positional cloning of *R* genes from large-genome crop species is still far from a routine procedure mainly due to low recombination frequencies and the high repetitive nature that are characteristic of most complex *R* loci (Hulbert *et al.*, 2001). In potato, the heterozygous genome is an additional complicating factor (Kanyuka *et al.*, 1999). To partially circumvent these obstacles, our local RGA approach analyzes genetic markers on BAC pools or super-pools, offering enough template complexity in PCR to allow most markers to be allelic- and locus-specific when compared with the hybridization method on single BAC filters. Although the RGA-specific primers (P_{SL8D}) were designed for the exclusive identification of the *I2* gene family, they were also designed to be allele- and locus-insensitive in order to include all candidate RGAs at the target region. Through association on BAC pools, we identified a subset of RGAs that were in coupling phase with and physically close to genetic markers linked to *R3a* and thus 'jump' rather than 'walk' into BACs carrying such RGAs. The success of the method depends on the marker saturation level at the target region and the capacity of the RGA-specific primers to

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amplify all the candidate RGAs at the target region. In this case, we benefited from the marker saturation provided by the ultra-high dense (UHD) AFLP potato map (Isidore *et al.*, 2003), as the same mapping population (SH × RH) was used to generate the UHD map and for the initial mapping of *R3a* (Huang *et al.*, 2004). In general, a bulked segregant analysis (Michelmore *et al.*, 1991) in combination with AFLP marker technology will generate adequate marker saturation of the *R* gene regions as the 'wild' *R* haplotype often differs a lot from the 'domestic' *r* haplotype, which is also the reason that they hardly recombine. The synteny at the target region between tomato and potato was beneficial to the RGA approach, despite extensive expansion of the *R3* complex locus compared to the syntenic *I2* complex locus.

Most of the molecularly characterized *R* genes belong to families of tightly linked genes and at many such complex loci, most paralogues appear to encode proteins similar to the functional *R* genes (Hulbert *et al.*, 2001). We demonstrated that all four paralogues of the *R3a* cluster are constitutively expressed (Figure 5), even in unchallenged plants. It is unclear whether these paralogues are capable of interacting with unknown elicitors or whether they are just relics of a recent 'birth-and-death' process (Michelmore and Meyers, 1998). Given the fitness cost of *R* gene expression (Tian *et al.*, 2003), unnecessary *R* genes should undergo a 'death' process. We also found many truncated paralogues, mutated via frame-shift (data not shown), or inserted by a retroelement (Figure 2d). The weaker expression of *I2GA-SH23-3* (Figure 5) might suggest that mutations accumulated in the promoter region can lead to a lower expression and thus eventually to the 'death' of the gene. Alternatively, the expressed paralogous proteins may play a role through heteroduplex formation with R3a, as inter- and intramolecular interactions between R protein domains may function as activation switches upon recognition of Avr elicitors (Moffett *et al.*, 2002).

Plants cannot move to escape their microbial environment. To combat disease, plants develop a sophisticated innate immunity system, where *R* genes play a central role (Dangl and Jones, 2001). Comparative genomics may provide insight into how diseases have resulted in differential evolution of *R* loci between closely related plant species. The potato *R3* region has undergone a significant physical expansion compared to the syntenic tomato *I2* region. This expansion may have resulted in functional diversification. For instance, at least 10 *I2*GAs have been found at the *R3b* region (S. Huang, H. Kuang, E. van der Vossen, V. Vleeshouwers, E. Jacobsen, B. Baker and R. Visser, unpublished data), suggesting *R3b* could also be an *I2*GA. The contrasting evolutionary fates of the ancient *I2-R3* complex locus in the closely related tomato and potato genomes are consistent with the opposite evolutionary potentials of the interacting pathogens (McDonald and Linde, 2002). *Fusarium oxysporum* f. sp. *lycopersici* is a soilborne fungus with low genotype diversity, whereas the late blight pathogen *P. infestans* is notorious for its ability to move and mutate (McDonald and Linde, 2002). The great evolutionary potential of *P. infestans* may have stimulated the interacting *R3* complex locus to expand its *R* gene repertoire, as supported by the observed physical expansion and the fact that two functionally distinct genes locate in the locus (Huang *et al.*, 2004). *Phytophthora infestans* also infects tomato, but genomic regions controlling late blight resistance show very limited overlap between tomato and potato (Brouwer *et al.*, 2004; Gebhardt and Valkonen, 2001; Grube *et al.*, 2000). Late blight resistance in the two closely related species is likely conferred by different loci that have evolved independently.

Plant disease resistance genes display two distinct evolutionary patterns contrasting at the rate of evolution (Kuang *et al.*, 2004). The late blight *R* gene *RB/Rpi-blb1* from *Solanum bulbocastanum* belongs to the type II (slow evolving) *R* gene class (Song *et al.*, 2003). However, the occurrence of sequence exchanges between paralogues (Figure S2), the multi-allelic nature of the locus (see below), high nucleotide identities between homologues (90–94%) and obscure allelic/orthologous relationships between the SH *R3* haplotype and other *S. demissum* haplotypes (H. Kuang, S. Huang, X. Wang, R. Visser and B. Baker, unpublished data) lead to the putative classification of *R3a* into the type I (fast evolving) class. It remains unknown why these two late blight *R* genes differ by the rate of evolution, but there are some hints. *RB/Rpi-blb1* is resistant to all tested races of *P. infestans* and provides resistance by reducing the infection rate (Song *et al.*, 2003; van der Vossen *et al.*, 2003), while *R3a* is a race-specific gene and displays a typical hypersensitive necrosis response (Huang *et al.*, 2004). *Solanum bulbocastanum* often grows under quite dry conditions (Hawkes, 1990), suggesting the absence of co-evolution with *P. infestans*, whereas *S. demissum* has co-evolved with the pathogen on the cool mountain forests in Toluca Valley of Mexico (Rivera-Pena, 1990), which was recognized as a center of diversity for *P. infestans*.

An intriguing question is why *S. demissum* appears to contain only easily broken *R* genes (Wastie, 1991) but displays durable resistance at the population level (Rivera-Pena, 1990). The polymorphism of parasite recognition capacity in a host population will restrict most isolates of the parasite to grow on most hosts (Hamilton *et al.*, 1990). Allelism is an efficient way of creating recognition polymorphism (Bergelson *et al.*, 2001), and we are currently investigating the multiple allelism of the *R3* complex locus. (S. Huang, V. Vleeshouwers, E. Jacobsen and R. Visser, unpublished data), which might be a natural mechanism of *S. demissum* to suppress late blight epidemics, similar to the concept of *R*-gene polycultures or multilines (Jones, 2001; Mundt, 2002). The isolation of *R3a* and characterization of the *R3* complex locus will facilitate the cloning of other

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alleles and thus provide a platform to test the *R*-gene polyculture concept in late blight disease management in commercial potato production.

Experimental procedures

Plant material

The F_1 population of SH83-92-488 (SH) × RH89-039-16 (RH) that segregates for both *R3a* and *R3b* was used for genetic mapping (Huang *et al.*, 2004). SW8540-025 is a recombinant that only carries *R3a*. The susceptible potato clones 1029-31 and cv. Desiree were used for genetic transformation.

Phytophthora infestans *isolates and inoculation*

Phytophthora infestans isolates 89148-09 (*Avr3a*, *Avr3b*), IPO-0 (*Avr3a*, *avr3b*), H30P04 (*avr3a*, *Avr3b*), and 90128 (*avr3a*, *avr3b*) were inoculated on *in vitro* plantlets (S. Huang and V. Vleeshouwers, unpublished data) or detached leaves (Huang *et al.*, 2004).

Genetic mapping

A total of 3000 SH × RH progeny were screened for recombinations in the *R3a* region using the flanking markers cLET5E4 and CT120 (Table 1A). The recombinants were used to determine the position of the *R3a* markers (Figures 1 and 2a). DNA isolation and analysis of molecular markers were described previously (Huang *et al.*, 2004).

Physical mapping

A 10-genome equivalent BAC library of the resistant parent SH, stored in 255 384-well microtiter plates, was screened with the *R3a* markers stepwise: first, positive superpools (1 superpool = 8 BAC pools) were identified; secondly, positive pools were identified from those positive superpools. Plasmid DNA was isolated using the standard alkaline-lysis protocol from pooled bacteria to produce 255 BAC pools. Screening of the BAC pools and identification of single positive BACs were performed as described previously (Rouppe van der Voort *et al.*, 1999).

Plasmids of single BACs were purified using MIDI-PREP columns (Qiagen, Hamburg, Germany). Sequences generated through BAC-end sequencing with SP6 or T7 primers were employed to design PCR primers to develop BAC end markers. The BAC end markers were named according to the BAC pool number followed by S (SP6) or T (T7) (Table 1B). In view of the repetitive nature of the *R3* complex locus, the overlap of two BACs was verified by: (1) *Hin*dIII restriction mapping, (2) non-selective AFLP fingerprinting using *Hin*dIII and *Mse*I/*Taq*I (Simons *et al.*, 1998), and (3) analysis of BAC end markers through T_m-gradient PCR, restriction with 24 frequent cutters, and sequencing of the PCR products. The length of overlapping between two BACs was determined by adding the sizes of co-migrating *Hin*dIII restriction fragments.

DNA sequencing and analysis

The DNA sequences of the BAC clones SH194C17 and SH23G23 (Figure 2d) were determined by using a shotgun sequencing strategy (van der Vossen *et al.*, 2000). Positions of putative genes were predicted using GENSCAN (Burge and Karlin, 1997) and GENEMARK (Lukashin and Borodovsky, 1998). Multiple sequence alignments were conducted by using CLUSTALX 1.81 (Thompson *et al.*, 1997) and IPS (Parniske *et al.*, 1997) were determined. Diversifying selection was investigated using PAML (Yang, 1997; Yang *et al.*, 2000). Models M7 and M8 in program 'codeml' of PAML were run for all *I2*GAs at the *R3a* cluster. Diversifying selection was confirmed using a likelihood-ratio test by comparing the likelihood calculated using models M8 and M7 (Yang *et al.*, 2000). Selection in each site of the *R3a* homologues was calculated using model M8.

Subcloning and transformation of I2*GAs*

Candidate *I2*GAs were subcloned into the binary vector pBINPLUS (van Engelen *et al.*, 1995) as described previously (van der Vossen *et al.*, 2003). Binary plasmids harboring the candidate genes were transformed to *Agrobacterium tumefaciens* strain AGL0 (Lazo *et al.*, 1991) and introduced into 1029-31 and cv. Desiree according to standard protocols (Visser *et al.*, 1991).

Transcript analysis of the R3a *cluster*

Total RNA was extracted from detached leaves of SH inoculated with *P. infestans* IPO-0, 90128, and water using TRIZOL (Invitrogen, Carlsbad, CA, USA). The mRNA was isolated from the total RNA samples using the OLIGOTEC kit (Qiagen). The 5′- and 3′-ends of the *R3a* transcript were determined by rapid amplification of cDNA ends (RACE) using the MARATHON kit (BD Bioscience, USA) with nested gene-specific primers (3RACE4-6 and 5RACE4-6, Table 1D). The cDNA for 5′ RACE was synthesized with the primer 5RACE0. PCR products were cloned into pGEM-T vector (Promega, Madison, WI, USA). For gene-specific RT-PCR (Table 1E), an aliquot of 0.1 μg mRNA from each sample was used to generate single-stranded cDNA using SUPERSCRIPT II (Invitrogen). Actin primers were used to monitor mRNA concentration (Table 1E). Genomic DNA of SH and RH and BAC subclones were used as control. Gene-specific amplification was confirmed by sequencing.

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Supplementary Material

The following material is available from http://www.blackwellpublishing.com/products/journals/suppmat/TPJ/TPJ2365/TPJ2365sm.htm

Figure S1. The distribution of codons under diversifying selection in *R3a*.

Figure S2. Sequence relationships between members of the *R3a* cluster.

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

genomic structure, functional diversity, and implications

Sanwen Huang

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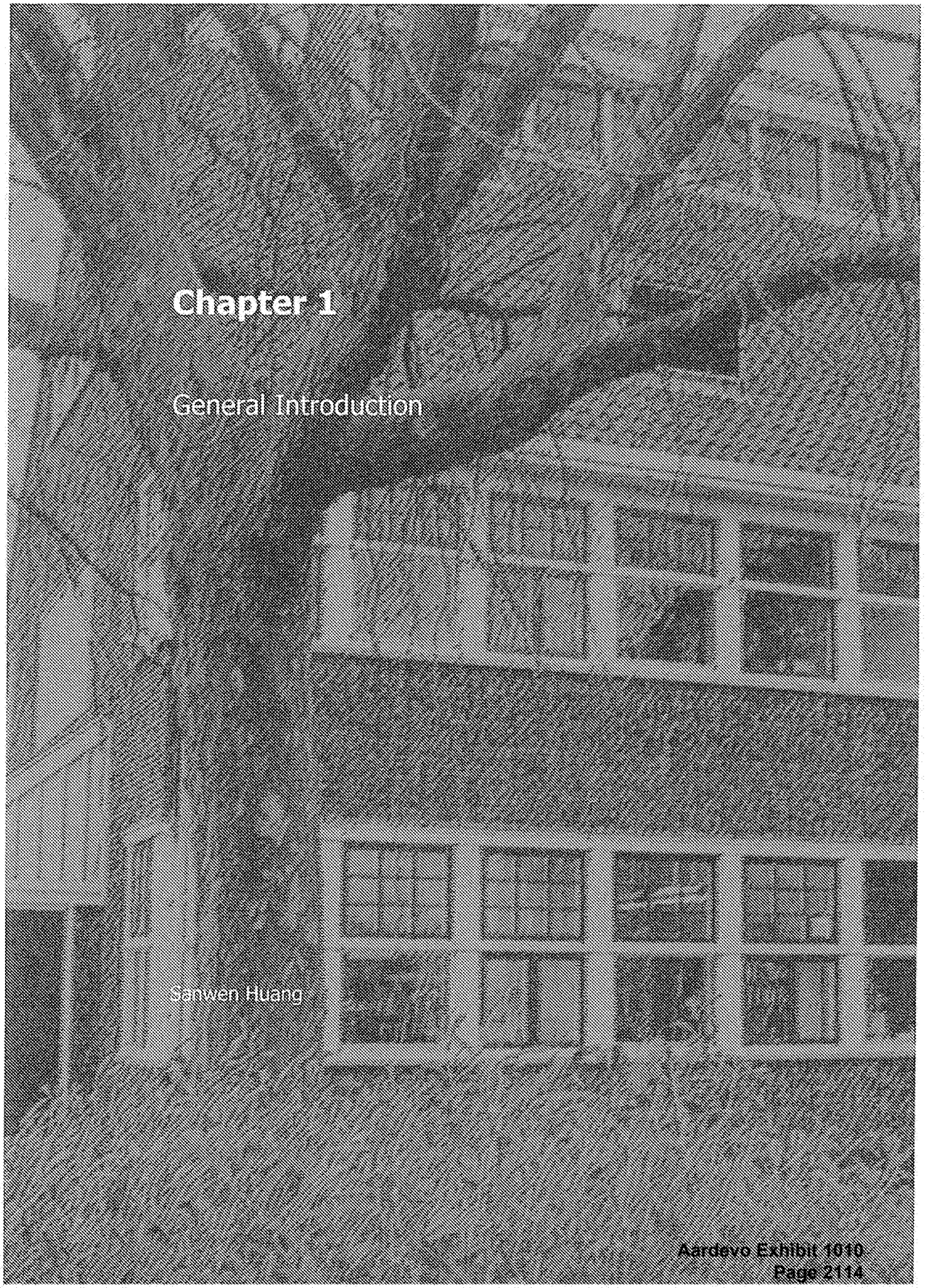
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Chapter 1

General Introduction

Sanwen Huang

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General introduction

Potato-the crop

Economical importance of potato

Potato (*Solanum tuberosum* L.) is the fourth most important crop and the most important non-cereal crop in the world, with an annual production of over 300 million tons. Its nutritional value was recognized by Western countries, and is now one of the staple foods in Europe and Northern America, with a per capita consumption of 86 kg/yr and 65 kg/yr, respectively. In recent years, the consumption in developing countries also increased: from 9 kg per capita in 1961-63 to 15 kg per capita in 1995-97. This was associated with a significant growth in production and planting area and with a modest increase in yield.

Potato is the number 1 crop of the Netherlands, occupying over 20% of the arable lands. The Netherlands plays a major role in the world trade of potato, particularly in seed potatoes. Nearly 80% of seed potato export is in the hands of Dutch companies. China is now the world's largest potato producer (Qu et al., 2004), reaching an annual production of 68 million tons with an average growth rate of 6.7% from between1980-83 and 2000-03. However, compared with the Netherlands, China's potato agribusiness is still in its infancy (Table 1). The yield is only 1/3 of that in the Netherlands. On average, each Chinese person only consumes 1/6 the amount of potato as a Dutch person. Although 22% of the potato production in China is used for processing, the products are mainly of low quality with a poor market value. Industries producing French fries, chips, and high quality starch products are just emerging. However, the low starting point offers enough space to grow for Chinese potato agribusiness along with the booming of the overall economy. Diversification of diets and westernalization of life style has created a great potential for the potato market. The younger generation is consuming more and more potato snacks. China's textile, papermaking, and food industries require a huge amount of potato starch and its derivatives, while China can only produce a small portion. If in the next 10 years the consumption of potato in China reaches the average level of the world, China will need to double its production. That is a great challenge, since arable land is decreasing. The only way is to improve the yield. This will offer great opportunities for countries like the Netherlands with a developed infrastructure for potato cultivation, storage, and processing. Transfer of cultivars, techniques and knowledge to China will have a high marginal benefit because requirement of these resources within developed countries is almost saturated.

Table 1. Some general data on potato in China, the Netherlands, and the world [a].

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	Production						Rank [b]
	2000-03			Average annual growth rate (1980-83~2000-03)			
	Product.	Area	Yield	Product.	Area	Yield	
	x1000 t	x1000 ha	t/ha	%	%	%	
China	68,250	4,654	14.7	5.7	3.4	2.6	5
Netherlands	7,226	165	43.8	0.9	0.9	0.0	1
World	318,139	19,388	16.4	0.4	0.1	0.3	4

	Utilization					Con-sump. per capita	Trade [c]			
	Food	Feed	Seed	Process.	Other		Imports		Exports	
	%					kg/yr	x1000 t	M$	x1000 t	M$
China	38	31	6	22	5	14	3.7	4	79.8	9.5
Netherlands	29	14	8	47	3	87	1,479	143	1,461	332.7
World	54	19	12	8	8	28	8,103	1,715	7,938	1,535

[a] Source: production and trade: FAOSTAT (2004); utilization and consumption: FAOSTAT (1998);
[b] In order of importance vs. 20 other major food crops on a fresh-weight basis;
[c] Table potatoes and seed only; does not include processed potato products.

Taxonomy of potato

Potato belongs to the botanical family Solanaceae that contains more than 3,000 species, most of which evolved in the Andean/Amazonian regions of South America. The family is the third most important plant family, ranked after the cereals and legumes, and includes other important crops such as tomato, tobacco, eggplant, and pepper. The Solanaceae genome is highly conserved (Gale and Devos, 1998). All *Solanum* species carry the same basic chromosome number (1x=12). Potato and tomato only differ by five inversions (Bonierbale et al., 1988).

It is assumed that potato has more related wild species than any other crop, with some 228 wild *Solanum* species recognized. The main cultivated germplasm for long-day conditions is derived from *S. tuberosum* Group Tuberosum, whereas *S. tuberosum* Group Andigena is adapted for short-day environments. In the tuber-bearing subsection of the genus *Solanum*, 19 series are classified ranging from diploid (2n=2x=24) to hexaploid (2n=6x=72) and distributed over an extraordinarily wide range of habitats, where they have become adapted to the local abiotic and biotic environments. The genes involved in pest and disease resistance in these wild relatives can be of use for potato breeding (Hawkes, 1994).

Potato genetics and breeding

The cultivated potato is an auto-tetraploid (2n=4x=48) with four sets of similar chromosomes (where n is the gametic chromosome number and x is the basic number). The wild tuber-bearing relatives form a polyploid series with species

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having 24, 36, 48, 60, and 72 chromosomes. More than 70% of them are diploids (2n=2x=24). The tetrasomic inheritance of the cultivated potato is infinitely more complicated than that of its 2x relatives. Fortunately, dihaploid plants (2n=2x=24) of tetraploids (2n=4x=48) can be obtained with ease from the cultivated tetraploids by means of anther culture or parthenogenesis (Hermsen and Verdenius, 1973; Ortiz and Peloquin, 1994). These dihaploids can be crossed with most of the wild diploid (2n=2x=24) species to capture their genetic diversity. Upon identification and characterization at the diploid level, desirable genes can be transferred into elite tetraploid gene pools through unreduced gametes (2n gametes) (Peloquin et al., 1999) and be used in commercial breeding programs.

Potato breeding involves tremendous efforts. A typical breeding program often starts with 100,000 seedlings and ends with two or three hopefully successful cultivars in more than 10 years (Bradshaw and Mackay, 1994). Two technologies can improve the efficiency of potato breeding. One is marker-assisted selection (MAS), another is genetically modified organism (GMO) technology.

The MAS strategy requires a well-established genetic map. The first generation of potato genetic maps was composed of restriction fragment length polymorphism (RFLP) markers (Gebhardt et al., 1991; Tanksley et al., 1992; Jacobs et al., 1995). The high intraspecific polymorphism of potato was exploited to construct the second generation of genetic maps based on amplified fragment length polymorphism (AFLP) markers (van Eck et al., 1995). This resulted in the construction of the Ultra-High Density (UHD) potato AFLP map (Isidore et al., 2003), on which more than 10,000 AFLP markers have been located by analysis of 130 F1 progeny from the cross of two diploid clones, SH83-92-488 (SH) and RH89-039-16 (RH) (http://www.dpw.wur.nl /uhd). The UHD map will serve multiple purposes ranging from resolving complex genetic traits into their single gene components and facilitating efficient gene introgression into commercial cultivars by marker assisted breeding to positional cloning of any potato gene of interest. The limitations are the rather time- and cost-consuming AFLP procedures and the access of the AFLP technique by breeding companies because it requires a license from the patent holder KeyGene. The third generation of genetic maps will be based directly on DNA sequences released from the potato genome sequencing consortium coordinated by Wageningen University, the tomato genome sequencing initiative coordinated by Cornell University (SOL, http://www.sgn.cornell.edu /solanaceae-project/), and the tobacco genome sequencing initiative coordinated by North Carolina State University. A genetic map saturated with simple PCR markers with high polymorphism can be expected to be one of the spin-offs of these genome sequencing programs.

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The GMO approach is more targetted, often focused on a single gene with known biological or biochemical function, involving direct modification of a single trait. This approach can either knock down a target gene or add a new gene. The most successful example in gene knockdown in potato is the manipulation of the granule-bound starch synthase (GBSS) gene, a gene involved in the synthesis of amylose in potato tubers, by using the antisense RNA technique (Kuipers et al., 1994). Potato is amenable for Agrobacterium-mediated genetic transformation (Visser et al., 1991). With the anticipated availability of a complete genome sequence, gene cloning from wild relatives will be expedited. The gene flow from wild species to elite cultivars (cvs.) via genetic transformation can save decades and circumvent problems such as linkage drag. The limitation of the GMO approach is the public concern about biosafety and food safety of GMO products. With the sophisticated marker-free technology (de Vetten et al., 2003), GMO will more likely be accepted by the society and will exhibit its great potential in potato breeding.

Phytophthora infestans-the pathogen & late blight-the disease

Potato late blight

Phytophthora infestans (Mont.) de Bary is the cause of potato late blight and is also known as the Irish potato famine fungus. In Latin, *Phytophthora* means plant destroyer, which exactly fits this notorious pathogen. Late blight totally destroyed Ireland's potato crop during 1845 and 1846, which resulted in the death of about one million people due to starvation and the emigration of another million (Bourke, 1991). Probably no other single plant pathogen caused such widespread human suffering. Today, late blight is still the No. 1 threat to potato production. World-wide losses in potato production caused by the disease have been estimated to cost $3 billion annually (Duncan, 1999). China produces 1/5 of the potato produced in the world, and we estimate that the loss to late blight in China will be about $600 million annually.

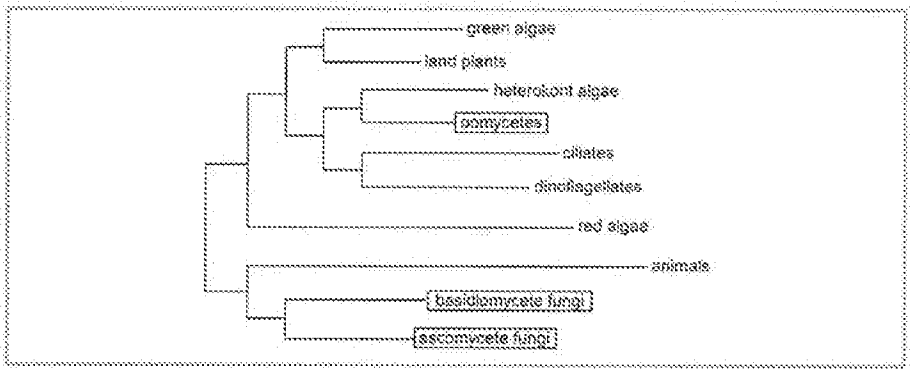
Phytophthora infestans is an oomycete

The genus *Phytophthora* belongs to the family *Pythiaceae*, in the class oomycetes. Although oomycetes have many fungus-like characteristics, they are not classified in the Kingdom Fungi. Studies of cell wall composition, metabolism, and rRNA sequences indicate that oomycetes are better classified with golden-brown algae in the Kingdom Protista (Fig. 1). Oomycetes and fungi have overlapping strategies

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but distinct genetic and biochemical mechanisms to interact with plants (Latijnhouwers et al., 2003).

Fig. 1. Phylogenetic tree showing the evolutionary realtionships between the major eukaryotic groups. The Oomycetes and the ascomycetous and basidiomycetous fungi are highlighted in grey. Note the evolutionary distance between the Oomycetes and the fungi. Reproduced from Kamoun et al. (1999).



The disease cycle of *P. infestans*

P. infestans is generally considered to be a specialized pathogen causing disease on leaves, tubers and fruits of potato or tomato crops, although natural infection of plants outside the genera Solanum have been reported (Erwin and Ribiero, 1996). The disease cycle of *P. infestans* is well studied. Initially, pathogenesis involves asexual growth of the pathogen. Infections often begin when sporangia land on the leaf surface. Under humid and cool conditions, zoospores are released from the sporangia, encyst, and produce a germ tube. The tip of the germ tube develops into an appressorium, which forms a penetration peg that goes through the cuticle and penetrates the underlying plant cell. An infection vesicle is produced in the epidermal cell and hyphae grow into the mesophyll cell layers both intra- and intercellularly. Occasionally, intracellular haustorial feeding structures are formed. After three to four days, *P. infestans* starts to grow saprophytically in the necrotised center of the growing lesion. Hyphae emerge through the stomata and sporangiospores are formed which produce numerous new sporangia mainly on the underside of the leaf. Infected foliage first becomes water-soaked and eventually turns black. Tubers become infected later in the season. In early stages, slightly brown or purple blotches appear on the skin. In humid soils the disease progresses quickly, and the tubers decay either before or after harvest.

P. infestans can also multiple via a sexual cycle. It is a heterothallic organism with two known mating types, A1 and A2. In response to hormones, male and female gametangia are formed. Haploid nuclei are generated in the

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gametangia. An A1 and an A2 nucleus fuse and an oospore containing one diploid nucleus is generated. The oospore matures rapidly and a thick wall is formed, which enables the oospore to survive in the soil for many years. After germination of the oospore, new progeny, either A1 or A2, is able to infect newly planted tubers, or stems and leaves which come into contact with the soil (Drenth et al., 1995).

Phytophthora infestans enters the genomics era

To be able to control late blight requires a better knowledge of the pathogen that causes it. Recently, quite some exciting technological advances and resource enrichment enabled us to zoom in on the pathogen with genomics tools (Kamoun et al., 2002; Latijnhouwers et al., 2003). These included the construction of molecular genetic linkage maps (van der Lee et al., 1997), construction of a BAC library (Whisson et al., 2001), EST sequencing (Kamoun et al., 1999b), whole genome sequencing of the related *P. sojae* (Kamoun et al., 2002), and the development of multiple functional genomics methods as genetic transformation of *P. infestans* (Judelson et al., 1991), internuclear gene silencing (van West et al., 1999), functional examination of *P. infestans* effectors in plants such as *Nicotiana benthamiana* using Agrobacterium or PVX mediated transient expression (Torto et al., 2003, Vleeshouwers et al., unpublished work).

These technical developments enabled the discovery and characterization of a few important *P. infestans* genes involved in pathogenesis and (a)virulence. The INF1 elicitin, a 10 kDa extracellular protein, induces a hypersensitive response (HR) in a restricted number of plants, particularly those of the genus *Nicotiana*. Using a gene silencing strategy to inhibit INF1 production, Kamoun et al. (1998) demonstrated that elicitin functions as an avirulence factor in the interaction between *N. benthamiana* and *P. infestans*. Similar strategies were adopted to show that the α and β subunits of the heterotrimeric G-protein of *P. infestans* are important for vegetative growth and sporulation and, therefore, for pathogenicity of this organism (Latijnhouwers and Govers, 2003; Latijnhouwers et al., 2004). Another interesting development is the study of avirulence genes of *P. infestans* that determine the compatibility or incompatibility with the host potato, which is discussed below.

Gene-for-gene model-the interaction

Plants can not move to escape their pathogens and, therefore, develop a sophisticated innate immune system to defend themselves, in which disease resistance genes or *R* genes play a major role (Dangl and Jones, 2001). Many

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plant-pathogen interactions, particularly those involving biotrophic parasites, are governed by specific interactions between pathogen *Avr* (avirulence) gene loci and corresponding plant *R* genes. When the *R-Avr* pair is present in both host and pathogen, the result is disease resistance or incompatibility. If either is inactive or absent, the result is susceptibility or compatibility. This crosstalk between host and pathogen was coined in the gene-for-gene model by Flor (1971), who extracted the concept from his work on the interaction between flax and flax rust. The direct implication of this important concept is that recognition events occur in the infection process.

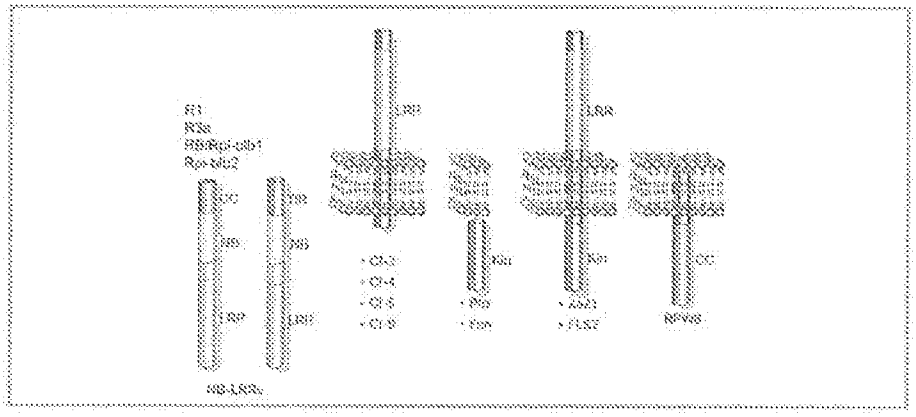


Fig. 2. Representation of the cellular location and structure of the five main classes of plant disease resistance proteins. Xa21 and Cf-X proteins carry transmembrane domains and extracellular LRRs. The RPW8 gene product carries a putative signal anchor at the N terminus. The Pto gene encodes a cytoplasmic Ser/Thr kinase (Kin), but may be membrane associated through its N-terminal myristoylation site. The largest class of R proteins, the NBS-LRR class, are presumably cytoplasmic (although they could be membrane associated) and carry distinct N-terminal domains. Reproduced from Dangl and Jones (2001). All cloned late blight R genes belong to a sub-class of NBS-LRR with coiled-coil (CC) domain.

The simplistic molecular model for the gene-for-gene concept would be that a protein encoded by an *R* gene can directly recognize an elicitor encoded by the corresponding *Avr* gene. Quite some *R* genes from model plants or crop species have been identified in the past 10 years. Despite the wide range of pathogen taxa and their presumed pathogenicity effector molecules, *R* genes encode only five classes of protein (Fig. 2) and the majority belongs to the nucleotide binding site plus leucine-rich repeat (NBS-LRR) class. LRR domains are found in diverse proteins and function as sites of protein-protein interaction. Although the characteristics of LRR domains favor the direct recognition model, there is hardly any experimental evidence except for one exception (Jia et al., 2000). As it generally goes in science, an alternative hypothesis had to be developed and this

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time it is the 'guard hypothesis' (Fig. 3). According to this model, an R protein plays as a guard to detect the potential exploitation of a host protein (guardee) by a pathogen effector (Avr). The model was firstly proposed to rationalize why the tomato Pto protein kinase requires the NBS-LRR protein Prf to activate defense upon the recognition of the AvrPto effector from the bacterial pathogen *Pseudomonas syringae* (Van der Biezen and Jones, 1998) and later was reinforced by studies on the model Arabidopsis-*P. syringae* pathosystem (Mackey et al., 2002; Axtell and Staskawicz, 2003; Mackey et al., 2003)

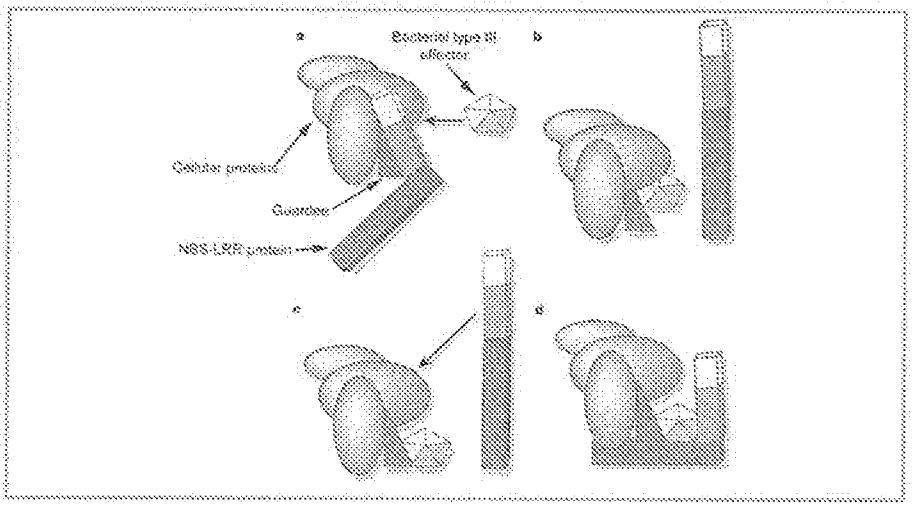


Fig. 3. Scenarios for the guard hypothesis for R-protein function. Reprod. from Dangl&Jones (2001).
a, A cellular complex of proteins, which includes both the 'guardee' molecule and an NBS-LRR protein (grey, shaded from the N terminus through NBS and LRR domains), is a target for a bacterial type III effector of disease.
b, Binding of the type III effector to its targets results in disassociation and activation of the NBS-LRR protein and thus disease resistance.
c, Alternatively, the NBS-LRR protein may not be part of the target complex until after type III effector binding.
d, Recruitment to the type III effector/target complex would then activate the NBS-LRR protein.

The gene-for-gene model also suits the potato-*P. infestans* pathosystem (Fig. 4). Exciting advances in studies on both *R* genes and *Avr* genes enable a better understanding of the crosstalk between the host and the oomycete, as described below.

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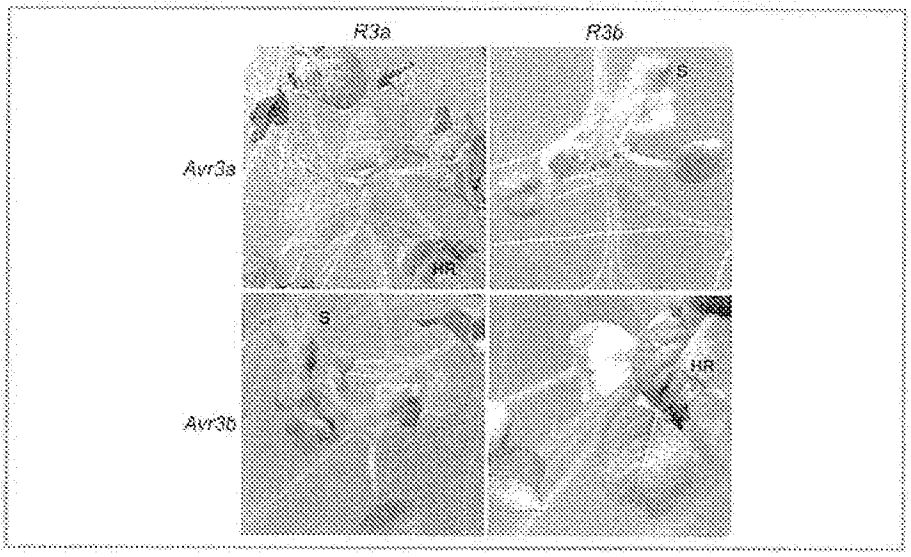


Fig. 4. The gene-for-gene interaction between potato and *P. infestans*. Phenotype of plants carrying *R3a* and *R3b* inoculated with *P. infestans* isolates carrying *Avr3a* and *Avr3b*. Localized hypersensitive necrosis (HR) and sporulating lesions (S) are observed on the incompatible and compatible interactions, respectively.

Potato late blight resistance and *R* genes

Type of resistance and mode of action

Disease resistance to *P. infestans* may occur at subspecies or variety level (race-specific) (Black et al., 1953) or at the species or genus level (nonhost resistance) (Kamoun et al., 1998). In another dimension, resistance may be a qualitative black-and-white phenotype or a quantitative one with partial reduction in disease severity. In all these types, HR plays an essential role (Kamoun et al., 1999a; Vleeshouwers et al., 2000). The general involvement of HR implies the occurrence of recognition during interaction of *P. infestans* and host cells. It remains unclear to what extent passive protection such as waxy cuticular 'skin' layer or preformed anti-microbial compounds is involved in the non-specific resistance to *P. infestans*. Interestingly, dysfunction of a cell wall glycosyl hydrolase, was recently shown to allow enhanced penetration of *P. infestans* to its nonhost Arabidopsis (http://www.arabidopsis2004.de/01_seiten 006_3_main_detail2.php?abs_id=0572).

Sources of resistance

The diversity of the tuber-bearing species genepool provides a rich resource of genes against various abiotic and biotic stresses. Among many others, *S. demissum* is the most exploited wild species for late blight resistance. This allo-hexaploid can directly cross with tetraploid *S. tuberosum*. Alternatively, the diploid *S. phureja*

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can be used as a bridge. Started almost a century ago, late blight *R* genes from *S. demissum* have been introgressed into potato cvs. (Müller and Black, 1952). It was estimated by Ross (1986) that more than 83% of cvs. of the former Federal Republic of Germany carry genes from *S. demissum*. Introgression of *R* genes from other species is also ongoing but seldom results in the deployment at the cultivar level. One of the reasons is the barrier in crossing, for example, a double bridge-crossing using *S. acaule* (2n=4x=48) and *S. phureja* (2n=2x=24) was conducted to introgress late blight *R* genes from *S. bulbocastanum* (2n=2x=24) into *S. tuberosum*, and in early 1970's this resulted in the ABPT clones (Hermsen and Ramanna, 1973). It was not until 2004 that the first potato cv. Biogold (Van Rijn BV) carrying gene(s) from the ABPT clones was released.

Late blight *R* genes

R genes are often genetically characterized as single dominant genes and functionally illustrated as race-specific determinants. Information on late blight *R* genes derived from wild species is listed in Table 2. Eleven resistance specificities (*R1-R11*) from *S. demissum* were characterized, while the inheritance of these specificities is not always known. *R1*, *R2*, and *R3* were mapped as single *R* loci on chromosome 5, 4, and 11, respectively. *R6* and *R7* co-localize with *R3*. *R4* is independently inherited from *R1*, *R2* and *R3* but its chromosomal position remains to be determined. The inheritance and position of *R5* and *R8-11* are unknown. Three *R* genes from *S. bulbocastanum* (Table 2) display a wide spectrum of resistance but await testing with more *P. infestans* isolates. Less studied are the *R* genes from *S. berthaultii*, *S. pinnatisectum* etc. Recently, four R genes have been cloned and they all encode coiled coil (CC)-NBS-LRR proteins. These include the *R1* (Ballvora et al., 2002) and *R3a* (**Chapter 4**) genes derived from *S. demissum* and the *RB/Rpi-blb1* (Song et al., 2003; van der Vossen et al., 2003) and *Rpi-blb2* (van der Vossen, personal communication) genes from *S. bulbocastanum*. These cloned *R* genes provide the starting point to study resistance mechanisms and to design novel approaches for late blight disease management.

Comparative genomics and *R* gene cloning

Comparative genomics investigates the similarity and differences in structure and function of genomes across taxa and is a powerful tool to transfer knowledge from model organisms into less studied ones. Full genome sequences of Arabidopsis (2000) and rice (Goff et al., 2002; Yu et al., 2002) and several other ongoing sequencing projects will offer unprecedented resources to study the evolution of sequences and functions of orthologous genes and to understand diversification and

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adaptation. A fundamental and practical question in comparative genomics is whether this vast amount of sequence information from model plant species will enable the cloning of genes with agronomic importance from crop species with larger genomes. Resistance to plant pathogens is an important crop trait that could benefit from sequencing of model species and facilitate gene cloning. This, however, will largely depend on the given plant family. Extensive loss of colinearity caused by segmental duplications, selective gene losses and significant genome reshuffling was discovered within crucifers and grasses (Gale and Devos, 1998; Paterson et al., 2000). Comparative analysis based on DNA sequences has revealed that disease resistance (*R*) loci may be evolving faster than the rest of the monocot and crucifer genome (Leister et al., 1998; Tarchini et al., 2000; Brueggeman et al., 2002; Hall et al., 2002), although a few phenotypically defined quantitative *R* loci (QRL) to the blast fungus appeared to be conserved between rice and barley (Chen et al., 2003). In contrast, the remarkable conservation of gene order (Bonierbale et al., 1988) makes the Solanaceae, the botanical family that includes many important crops such as tomato and potato, an attractive subject for comparative genomics. Low-resolution comparative mapping indicated that *R* loci may be positionally conserved within Solanaceae (Leister et al., 1996; Grube et al., 2000; Pan et al., 2000). Several *R* gene hot spots have been recognized with resistance to a diversity of pathogens (Grube et al., 2000; Gebhardt and Valkonen, 2001).

Currently, positional cloning is the only feasible approach in *R* gene isolation in potato. However, this approach has often been hindered by the fact that *R* loci have an extremely low frequency in recombination. Moreover, the heterozygous genome of potato makes chromosome walking very complicated. Comparative genomics can partially alleviate these difficulties by providing a clue of the sequence identity of the genes and, therefore, makes the approach more targeted. To date, comparative genomics has lead to the isolation of two late blight *R* genes. The first one is the *R3a* gene from the *R3* complex locus (**Chapter 4**), which has an orthologous relationship with the tomato *I2* locus conferring resistance to Fusarium wilt (Ori et al., 1997; Simons et al., 1998). Another one is the *S. bulbocastanum Rpi-blb2* gene (van der Vossen, *personal communication*), syntenic to the tomato *Mi* gene that expresses resistance to root-knot nematodes.

Avirulence and *avr* genes

Genetic maps of *Avr* genes

The existence of two mating types (A1 and A2) of *P. infestans* enables the sexual crossing of two isolates that differ by in their avirulence phenotype. Genetic

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linkage maps have been constructed by analysis of progeny from such crosses with molecular markers. Six *Avr* genes interacting with known *S. demissum R* genes have been mapped (van der Lee et al., 2001). *Avr1* that interacts with the *R1* gene was mapped on linkage group (LG) IV, *Avr2* on LG VI, *Avr3-Avr10-Avr11* on LG VIII, and *Avr4* on LG A2-a. It is worthwhile to mention that the functional definition of *Avr* genes are based on *R* gene differentials. If a single *R* gene differential carries more than one *R* genes, the segregation of the corresponding avirulence phenotype should reflect this fact. For example, avirulence to the *R3* differential is controlled by two loci in some *P. infestans* crosses (Spielman et al., 1989). This is well consistent with our finding that the *R3* locus consists of two functionally distinct genes (**Chapter 3**). Also the clustering of *Avr3-Avr10-Avr11* was unexpected, to which we offer an explanation (**Chapter 5**).

Table 2. Major *R* genes derived from wild relatives of potato, with species of origin, chromosomal position, and race specificity.

R gene	Species	Chrom.	Race-specificity	Ref.
R1	*S. demissum*	5	Yes	Ballvora et al., 2002
R2		4	Yes	Li et al., 1998
R3a, R3b		11	Yes	Huang et al., 2004
R4		unknown	Yes	Müller and Black, 1952
R5		11	Yes	Chapter 5
R6 [a]		11	Yes	Chapter 5
R7		11	Yes	Chapter 5
R8		11	Yes	Chapter 5
R9 [a]		11	Yes	Chapter 5
R10		11	Yes, partial	Chapter 5
R11		11	Yes	Chapter 5
RB/Rpi-blb1	*S. bulbocastanum*	8	Wide-spectrum	Song et al., 2003; van der Vossen et al., 2003
Rpi-blb2		6	Wide-spectrum	Van der Vossen, personal com.
Rpi-blb3		4	unclear	Park et al., personal com.
Rpi-ber1	*S. berthaultii*	10	Yes	Ewing et al., 2000
Rpi1	*S. pinnatisectum*	7	Unclear	Kuhl et al., 2001
Rpi-mcd1	*S. microdontum*	5	Unclear	Brigneti et al. Poster No. 588 of Plant and Animal Genomes XII, San Diego, 2004
Rpi-moc1	*S. mochiquense*	9	Unclear	
Rpi-neo1	*S. neorossii*	4/7	Unclear	
Rpi-oka1	*S. okadae*	9	Unclear	
Rpi-oka2		4	Unclear	

[a] *R6* and *R9* differentials have more than one *R* locus, as shown in chapter 5.

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The map-based cloning was adopted to isolate some of the mapped *Avr* genes (Whisson et al., 2001). However, the difficulty in generating large segregating F1 populations from *P. infestans* hampers certainly influence the feasibility of this approach. Transcriptional profiling of virulent and avirulent F1 progeny offers a suitable alternative for map-based cloning. Jiang, Guo, and Govers (personal communication) characterized cDNA-AFLP fragments specific for avirulent isolates and with these fragment full length candidate *Avr* genes are being isolated. In addition, high-throughput functional genomics approaches such as the binary PVX method (Takken et al., 2000) and intraspecific comparative genomics (Bos et al., 2003) are adopted to clone *Avr* genes. The latter enabled the isolation of *Avr3a* gene from *P. infestans* (Armstrong et al., submitted).

To date, two race-specific avirulence genes of oomycetes have been well characterized. Shan et al. (2004) recently used map-based cloning to identify a locus in *Phytophthora sojae* containing two genes, *Avr1b-1* and *Avr1b-2*, which are required for avirulence on soybean plants carrying the resistance gene *Rps1b*. *Avr1b-1* encodes a small secreted protein. In some isolates of *P. sojae* virulent on *Rps1b*-containing cultivars, the *Avr1b-1* gene had numerous substitution mutations indicative for a strong divergent selection. *Avr1b-2* is required for the accumulation of *Avr1b-1* mRNA. *Avr1b-2* was genetically mapped to the same BAC contig as *Avr1b-1*. The *Avr1k* gene, required for avirulence on soybean cultivars containing *Rps1k*, was mapped at the same interval as *Avr1b-1*. The clustering of *Avr* genes resembles the clustering of *R* genes observed in many *R* loci. It is interesting to determine whether this feature of genomic organization of *Avr* genes favors the generation of diversity to enable the pathogen to circumvent the *R* genes and at the same time maintain the virulence function of *Avr* genes. Interestingly, the avirulence gene *ATR13* in *Hyaloperonospora parasitica* (formally *Peronospora parasitica*) exhibits extreme levels of amino acid polymorphism (Allen et al., 2004), as the corresponding *RPP13* resistance gene in *Arabidopsis thaliana* does. Evidence of diversifying selection detected in both partners suggests that a constant coevolutionary conflict has undergone at these interacting loci in the host and pathogen.

Control of late blight

P. infestans ranks highest in the evolutionary potential (McDonald and Linde, 2002). It has a mixed reproduction system and produces sexual outcrossing and asexual spores, which comprise extremely high genotypic diversity. It produces airborne asexual spores, and therefore has a high potential for genotype flow. The high evolutionary potential of the pathogen makes it difficult to control. Single-

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dominant host *R* genes, when deployed separately, have been rapidly overcome (Wastie, 1991). Chemical control is costly to farmers in developing countries and also raises environmental concerns in developed countries. Furthermore, the pathogen can mutate itself to become resistant to certain chemicals such as metalaxyl, a phenylamide used to control *Phytophthora* (Davidse et al., 1983). Biological and cultural control measures, such as antagonistic fungi, sanitation, and crop rotation can sometimes be effective, but the effect remains are limited due to difficulty in application. Overall, host resistance in combination with chemical control is at present the only available and relatively successful strategy, but this is limited to potato production in Western countries.

Typical "boom-bust" cycles were observed in deployment of the *S. demissum R* genes in commercial potato production. A cultivar with a new *R* gene provides a complete protection to the pathogen and its acreage will boom. However, the pathogen will mutate itself to overcome the *R* gene and infect the cultivar carrying it, causing the cultivar to bust. *R1*, *R2*, *R3*, and *R4* genes have been deployed separately but were overcome by virulent strains of *P. infestans* (Müller and Black, 1952). Isolates that can infect all the 11 resistance specificities were also reported but not frequently observed (Malcolmson, 1969). The 'boom-bust' cycles observed in the potato-*P. infestans* system is a rather general rule for gene-for-gene pathosystems in monoculture (Pink and Puddephat, 1999). To avoid such cycles, there are virtually two alternatives in *R* gene deployment: *R* gene pyramiding and *R* gene polyculture or multiline. Pyraming is the simultaneous deployment of several *R* genes in the same cultivar and polyculture or multilines is a mixture of cultivars with different *R* genes in the same genetic background. Pyramiding is the strategy adopted by potato breeders, but so far has only resulted in the release of a few cultivars, for instance the Dutch cv. Escort that contains *R1*, *R2*, *R3* and *R10*. To develop a cultivar with multiple *R* genes through traditional breeding is very time-consuming. *R* gene polyculture or multiline is also difficult to accomplish in potato through conventional breeding, since unlike self-crossing crops such as tomato or rice, potato is an outcrossing tetraploid that seriously suffers from inbreeding (Ross, 1986). So far the GMO approach is the only approach to efficiently implement *R* gene pyramiding or polyculture in potato. However, either strategy requires more cloned *R* genes than the four genes presently available.

Scope of this thesis

To understand the mechanism underlying the interaction of potato and *P. infestans* and to deploy *R* genes by means of pyramiding or polyculture for late blight

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control, we need to molecularly isolate at least a dozen or so *R* genes. This research describes the discovery and characterization of the major late blight resistance complex *R3* at the distal end of chromosome 11 in potato.

We applied a map-based cloning strategy in combination with comparative genomics to clone *R* alleles of this locus. This approach requires analysis of a large segregating population. For this purpose, we first designed a high throughput disease testing method using *in vitro* seedlings (**Chapter 2**). The assay exploits the amenability of potato for tissue culture and the suitability of the *in vitro* environment for late blight disease development. In two experimental setups, the specificity and reliability of the new method was compared with the well-established detached-leaf assay. The new technique was found to be a quick, space-effective, and accurate assay and can be a new on-hand tool for investigation of the qualitative interaction between potato and *P. infestans*.

Using the combination of fine-mapping and accurate disease testing with specific *P. infestans* isolates, we detected that the *R3* locus is composed of two genes, with distinct specificities (**Chapter 3**). The two genes, *R3a* and *R3b*, are 0.4 cM apart and both have been introgressed from *S. demissum*, the 'donor' species of most characterized race-specific late blight *R* genes. A natural recombinant between *R3a* and *R3b* was discovered in one accession of *S. demissum*.

To isolate the *R3a* gene, we used genomic information from the model Solanaceous plant tomato (**Chapter 4**). Comparative analyses of the *R3* region with the corresponding *I2* region in tomato suggested that this is an ancient locus involved in plant innate immunity against fungal and oomycete pathogens. However, *R3* has evolved after divergence from tomato and the locus experienced a significant expansion in potato without disruption of the flanking colinearity. This expansion may have resulted in an increase in the number of *R* genes and functional diversification, and might reflect the co-evolutionary history between *P. infestans* and *Solanum demissum*.

There is more on the *R3* complex locus! We demonstrated that except *R1*, *R2* and *R4*, all other characterized resistance specificities (*R5-11*) contain allelic versions at the *R3* complex locus (**Chapter 5**). That explains why only *R1-4*, and *R10* could be used in potato cultivars, since the others are allelic to *R3*. This unexpected discovery reveals that the potential for resistance breeding is not fully explored and implicates that the multi-allelism of the *R3* locus could be a putative natural mechanism for late blight control in the wild populations of *S. demissum*.

In the general discussion (**Chapter 6**), we recapitulate the results and discuss their further implications in late blight resistance breeding in potato.

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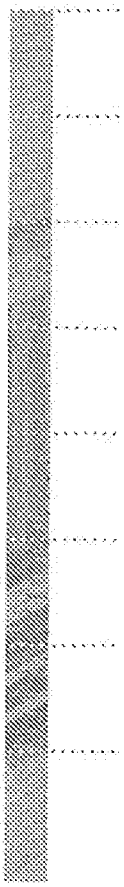
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Chapter 2

An Accurate *in Vitro* Assay for High-throughput Disease Testing of *Phytophthora infestans* in Potato

Sanwen Huang, Vivianne G.A.A. Vleeshouwers, Richard G.F. Visser and Evert Jacobsen

Submitted



An accurate *in vitro* assay for high-throughput disease testing of *Phytophthora infestans* in potato

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Abstract

***In vitro* inoculation was developed as a routine disease testing method to study the gene-for-gene interaction in the potato (*Solanum tuberosum*)-*Phytophthora infestans* pathosystem. The assay exploits the amenability of potato for tissue culture and the suitability of *in vitro* environment for late blight disease development. In two experimental setups, the specificity and reliability of the new method was compared with the well-established detached-leaf assay. First, four *P. infestans* isolates were tested for avirulence on a set of *R* gene differentials. The two methods gave identical conclusions on avirulence profiles of all isolates. Second, a population of 93 clones was phenotyped for segregation of two closely linked and functionally distinct genes -*R3a* and *R3b*- in the *R3* locus. For each clone both methods resulted in phenotypic scorings that were in perfect agreement. Furthermore, the phenotyping of the population was fully consistent with the genotyping obtained from analysis of molecular markers that flank each gene. The new technique was found a quick, space-effective, and accurate assay and can be a new on-hand tool for investigation of the qualitative interaction between potato and *P. infestans*.**

Introduction

Late blight, caused by *Phytophthora infestans* (Mont.) de Bary, remains the most devastating disease of potato (*Solanum tuberosum* L.) (Fry and Goodwin, 1997). Many resistance (*R*) genes from wild relatives have been introgressed into the cultivated potato via sexual or somatic hybridization (Black et al., 1953; Wastie, 1991; Helgeson et al., 1998). Most introgressed *R* genes are so-called major genes, i.e. they display a strong resistance response. Also minor genes for resistance occur in wild *Solanum* germplasms (Colon and Budding, 1988), and those confer a weaker or partial resistance. The interaction between potato and *P. infestans* follows the gene-for-gene model (Black et al., 1953; Flor, 1971; van der Lee et al., 2001). Several major *R* genes derived from wild species of potato and *Avr* genes in the oomycete pathogen were molecularly mapped (Gebhardt and Valkonen, 2001; van

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der Lee et al., 2001). Recently, three *R* genes have been cloned and were predicted to encode R proteins with coiled-coil, nucleotide-binding site, and leucine-rich repeat domains. The *RB/Rpi-blb1* gene derived from *S. bulbocastanum* confers a race-non-specific resistance to *P. infestans* (Song et al., 2003; van der Vossen et al., 2003). The *R1* and *R3a* genes are both derived from *S. demissum* and confer race-specific resistance (Ballvora et al., 2002, Huang et al., **Chapter 4**). The *R3a* gene and its closely linked but functionally distinct "twin" gene, *R3b*, explain the resistance specificity conferred by the *R3* locus. Several labs plan to isolate more late blight *R* genes using a map-based cloning strategy. This strategy often requires to reliably phenotype a large segregating population for creating a high-resolution genetic map. For this purpose, an accurate and high-throughput disease testing method is needed.

Many methods have been described to evaluate foliar late blight resistance, including field tests, whole-plant greenhouse assays (Stewart et al., 1983), and laboratory tests on detached leaves (Lapwood, 1961; Vleeshouwers et al., 1999), leaflets (Malcolmson, 1969; Umaerus and Lihnell, 1976) or leaf discs (Hodgson, 1961). The detached leaf assay is considered a reliable method for quantification of both major- and minor-gene resistances and is extensively used in laboratory studies (Vleeshouwers et al., 1999). However, for solely monitoring major genes, such extensive quantitative assays are not necessary and therefore, a quicker test should be feasible. Plants for detached leaf assays have to be grown in the greenhouse six to eight weeks before inoculation. Another disadvantage of greenhouse-grown plants is that the response to *P. infestans* of potato leaves can be affected by other diseases, such as powdery mildew, that is frequently observed on potato leaves in the greenhouse. Especially in high throughput genomics research, a faster testing assay is desirable.

Although potato is one of the most amenable plants for tissue culture and the high humidity and controllable temperature of the *in vitro* environment are suitable for disease development of *P. infestans* (Tegera and Meulemans, 1985), to date no *in vitro* experiment has been reported to study the specificity involved in the gene-for-gene interaction of potato and *P. infestans*. In this study we describe the development of *in vitro* inoculation as a routine method to study the interaction of *R* genes from potato and *Avr* genes from *P. infestans*. Using detached-leaf assay as a control, we assessed the specificity and reliability of *in vitro* inoculation in two experimental designs: (i) avirulence profiling of several *P. infestans* isolates using a set of *R* gene differentials; (ii) phenotyping of a population that segregates for the *R3* locus and confirmation by molecular marker analysis. The results from both experiments proved that *in vitro* inoculation could serve as an efficient and reliable

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alternative for the detached-leaf assay when major resistance genes and their cognate avirulence genes are concerned.

Table 1. The plant material used in this study and their known *R* genes to *P. infestans*.

Clone	Known *R* gene	Identity
Ma*R0* [a]	None	potato cv. Bintje
Ma*R1*	*R1*	CEBECO 43154-5
Ma*R2*	*R2*	CEBECO 44158-4
Ma*R3*	*R3a, R3b* [b]	CEBECO 4842-1
Ma*R4*	*R4*	CEBECO 4431-5
Ma*R5*	*R5*	Black 3053-18
Ma*R6*	*R6*	Black XD2-21
Ma*R7*	*R7*	Black 2182 ef (7)
Ma*R8*	*R8*	Black 242A a (5)
Ma*R9*	*R9* or *R1R2R3R9* [c]	Black 2573 (2)
Ma*R10*	*R10*	Black 3681 ad (1)
Ma*R11*	*R11*	Black 5008 ab (6)
RH89-039-16 (RH)	None	Paternal clone of the RH x SH *R3* mapping population
SH83-92-488 (SH)	*R3a, R3b*	Maternal clone of the RH x SH *R3* mapping population

[a] The Dutch differential set for profiling virulence of *P. infestans* isolates is designated as Ma*R0-R11*. Ma*R1*-Ma*R4* were developed by Dr. C. Mastenbroek, the other *R* differentials are identical to the Scottish differential set, developed by Black and colleagues (1953, 1966).
[b] Huang et al. (2004)
[c] Malcolmson and Black (1966)

Results

Determination of inoculum concentration *in vitro*

Inoculum concentration is an important factor that can influence the disease development (Stewart, 1990). An optimal inoculum concentration for *in vitro* conditions has to be determined. In a concentration serial experiment with *P. infestans* isolate IPO-0 *in vitro*, we inoculated zoospore suspensions of 0.5, 1, 2.5, 5 and 10 x 10^4 spores/ml on the resistant SH83-92-488 (SH, Table 1) and susceptible Bintje and RH89-039-16 (RH, Table 1). The inoculum concentration of 0.5 x 10^4 spores/ml resulted in suboptimal disease development for Bintje and RH. At the concentration of 10 x 10^4 spores/ml, SH gained intensive sporulation on inoculated leaves as saprophytic growth on the HR-caused necrotic tissue, and lesions spread to uninoculated leaves. Occasionally, spores dropped and mycelium started growing on the medium (Fig. 1). The intermediate concentration gave a clear phenotypic separation between resistant and susceptible genotypes. We thereafter chose

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2.5×10^4 spores/ml as the standard inoculum concentration for *in vitro* inoculation, half the amount as for detached leaves.

The first disease symptoms or defense responses on *in vitro* plantlets were generally visible at three to five dpi, depending on the interacting potato genotype and *P. infestans* isolate. The final scoring took place at six to eight dpi when a clear distinction between susceptible and resistant genotypes could be observed.

Compatible and incompatible interaction phenotypes on *in vitro* plantlets

In a compatible interaction, the inoculated leaves first turned to the abaxial side and lesions could be observed on the adaxial side of the inoculated leaves at three to five dpi. The lesions gradually spread across the entire inoculated leaves, to their petioles, to stems, to uninoculated leaves, and eventually the whole plantlets (Fig. 1). At six to eight dpi, depending on the level of susceptibility of the potato genotype and the agressiveness of the *P. infestans* isolate, compatible interactions were scored as interaction class 1 (spreading lesion with massive sporulation) or class 2 (spreading lesion with no or little sporulation). In both classes, the degradation of stems often caused the dropdown of the upper part of the plantlets and the spreading lesions eventually caused the death of the inoculated plantlets within two weeks.

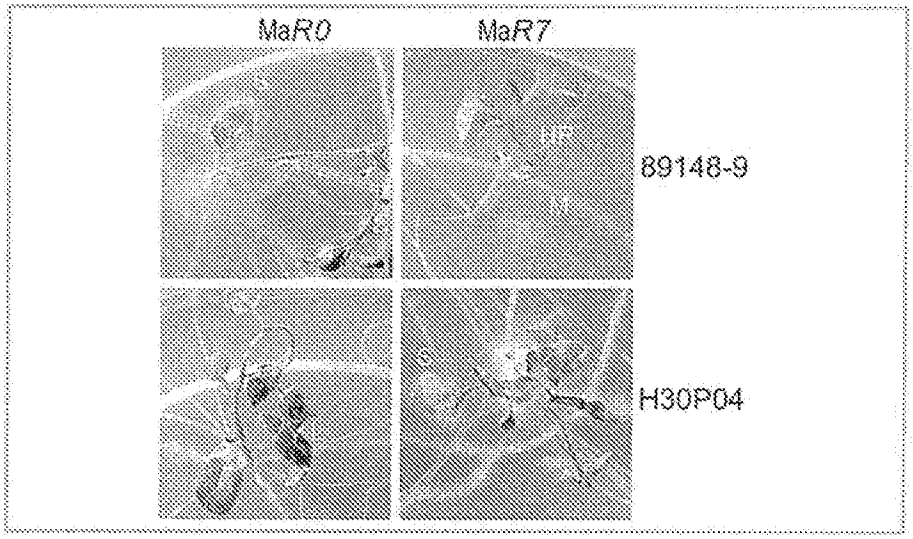


Fig 1. Compatible and incompatible interaction observed on *in vitro* plantlets. The *in vitro* plantlets of Ma*R0* and Ma*R7* inoculated with *P. infestans* isolates 89148-9 (race 0) and H30P04 (race 3a.7) at six dpi. A race-specific resistance response occurred on Ma*R7* leaves upon inoculation with isolate 89148-9 (HR). A small plug of mycelium (M) is growing on the surface of the medium. Extensive sporulation (S) or massive lesion (L) development was noted on the three compatible interactions.

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The phenotype of an incompatible interaction often appeared at two to four dpi. Plantlets showing no symptom or localized HR-like necrosis were scored as class 5 (Fig. 1), while those with trailing HR necrosis but limited to the inoculated leaf were scored as class 4.

Rarely, intermediate phenotypes between compatible and incompatible ones were observed, such as either sporulation on inoculated leaves or lesions spreading to only the upper part of the plantlets without sporulation. These interaction phenotypes were scored as class 3.

A typical differential interaction between *P. infestans* and potato was observed on *in vitro* plantlets (Fig. 1). The combination of *R7* and *Avr7* displayed a resistance phenotype with localized HR necrosis, while the other three combinations showed susceptible phenotypes, either as massive sporulation or as spreading lesion with little sporulation (Fig. 1). This result was perfectly consistent with the result obtained by using detached-leaf assay with the same potato clones and *P. infestans* isolates (data not shown).

Table 2. Avirulence profiling of *P. infestans* isolates GER8601, 99018, 91001, and 89148-27 with both *in vitro* (I) and detached leaf (D) assays. The phenotype and DIs (in parenthesis) of all differential-isolate combinations are shown and conclusions on the avirulence profiles are made.

	GER8601		99018		91001		89148-27	
Differential	I	D	I	D	I	D	I	D
MaR0	S (1)	S (1)	S (1)	S (1)	S (1)	S (1)	S (1)	S (1)
MaR1	S (1)	S (1)	S (1)	S (1)	R (5)	R (5)	R (5)	R (5)
MaR2	R (5)	R (5)	R (5)	R (5)	R (5)	R (5)	R (5)	R (5)
MaR3	R (5)	R (5)	R (5)	R (4)	R (5)	R (5)	S (1)	S (1)
MaR4	R (5)	R (5)	S (1)	S (1)	R (5)	R (5)	R (5)	R (5)
MaR5	R (5)	R (5)	R (5)	R (5)	R (5)	R (5)	R (5)	R (5)
MaR6	R (5)	R (5)	R (5)	R (5)	R (5)	R (5)	R (5)	R (5)
MaR7	R (5)	R (5)	R (5)	R (4)	S (2)	S (1)	S (1)	S (1)
MaR8	R (5)	R (5)	R (5)	R (5)	R (5)	R (5)	R (5)	R (5)
MaR9	R (5)	R (5)	R (5)	R (5)	R (5)	R (5)	R (5)	R (5)
MaR10	R (5)	R (5)	R (5)	R (4)	R (4)	R (5)	R (5)	R (5)
MaR11	R (4)	R (5)	nd	R (4)	R (5)	R (5)	R (4)	R (5)
Conclusion	race 1	race 1	race 1.4	race 1.4	race 7	race 7	race 3a.3b.7	race 3a.3b.7

Avirulence profiling

The avirulence profiles of four *P. infestans* isolates GER8601, 99018, 91001, and 89148-27 were determined on both detached leaves and *in vitro* plantlets of the differential set (Table 2). Scoring was conducted at both quantitative level using disease index (DI) and qualitative level using three classes: susceptible (S, DI ≤ 2.0),

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questionable (Q, 3.0 < DI < 4.0), and resistant (R, DI ≥ 4.0).

Identical conclusions were drawn for all four isolates, thus for qualitative resistance scoring, the *in vitro* vs. detached leaf results are fully matching (Table 2). The data were also analyzed quantitatively. Identical scores were obtained for each isolate with the two methods for most differentials, and only in a few combinations, minor quantitative differences were found in DIs. Of these, three gave a higher DI on detached leaves than on *in vitro* plantlets and five were the opposite, not suggesting a directional bias between the methods. Statistical analysis on DIs revealed that there was no significant difference between the two methods (R=0.971 for regression coefficient analysis and P=0.710 for two tailed T test).

Phenotyping and genotyping of the *R3* locus on a segregating population

The detached leaves and *in vitro* plantlets of SH and RH, the parental line of the *R3* segregating population, were inoculated more than ten times with two diagnostic *P. infestans* isolate IPO-0 (3b) and H30P04 (3a.7). Consistently, SH displayed a typical localized HR reaction that was visible at three dpi, while RH obtained intensive sporulation at five dpi, confirming our previous observation that SH carries both *R3a* and *R3b* and RH is susceptible (Huang et al., 2004). This clearly demonstrated that the *R3a* and *R3b* genes in SH are active in both detached leaves and *in vitro* plantlets.

We then investigated the reliability of the *in vitro* assay by phenotyping the *R3a* and *R3b* resistance using an SH x RH population of 93 plants. *In vitro* plantlets of the entire population and a subset of 33 plants were inoculated with *P. infestans* isolate IPO-0 and H30P04, respectively. For comparison, the entire population was phenotyped for both isolates with the detached leaf assay. To simplify the scoring, only qualitative scoring were conducted on the population using the same criteria as with avirulence profiling. In the population, 59 individuals were susceptible and 33 were resistant to both isolates. One plant (Table 3, progeny type 2) displayed a differential response both on detached leaves and *in vitro* plantlets. In all 126 observations, none gave a difference between the two methods, indicating that *in vitro* inoculation is reliable in phenotyping a large number of individuals (Table 3).

To confirm the result of phenotyping, we genotyped the whole population with the molecular markers cLET5E4 and GP185 flanking *R3a* and PCAMATA_4 and EATCMAGC_15 flanking *R3b* (Huang et al., 2004). No recombination was found between cLET5E4 and GP185 or between PCAMATA_4 and EATCMAGC_15 in the population, indicating that cLET5E4 and GP185 were fully diagnostic for the presence of *R3a*, and so were PCAMATA_4 and EATCMAGC_15

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for *R3b*. All 59 plants that were susceptible to the two isolates lacked the 'resistance' alleles of the four markers, while these 'resistance' alleles were present in all 33 plants that were resistant to the two isolates (Fig. 2). Marker analysis also confirmed the differential response of the single recombinant plant as being associated with the absence of the 'resistance' alleles on the *R3a* flanking markers and the presence of the 'resistance' alleles on the *R3b* flanking markers (Table 3, Fig. 2). These data clearly demonstrated that the *in vitro* assay could reliably phenotype a large segregating population and accurately display recognition specificities of potato *R* genes to the interacting oomycete pathogen.

Table 3. The SH x RH *R3* population was inoculated with *P. infestans* isolate IPO-0 and H30P04 using both the detached-leaf assay (D) and the *in vitro* assay (I). Parental clones RH and SH were included in the analysis. The individuals of the population were classified into three progeny types (PT), and the number of individuals within each PT is indicated.

Plant	*P. infestans* isolate				Number
	IPO-0 (3b)		H30P04 (3a,7)		
	D	I	D	I	
RH	S	S	S	S	
SH	R	R	R	R	
PT1	S	S	S	S	3
	S	S	S	nd	56
PT2	S	S	R	R	1
PT3	R	R	R	R	29
	R	R	R	nd	4
Total					93

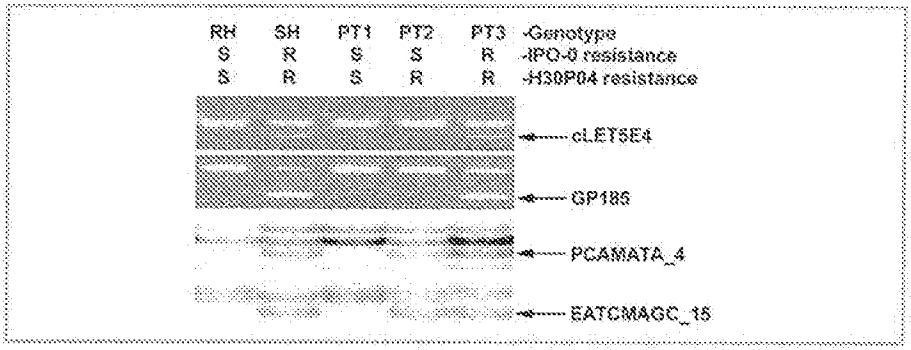


Fig 2. Genotyping of the three progeny types (PT) in the SH x RH *R3* mapping population and the parental clones RH and SH with four molecular markers. cLET5E4 and GP185 are flanking *R3a* and PCAMATA_4 and EATCMAGC_15 are flanking *R3b*. The markers are indicated at the right with an arrow pointed to the polymorphic band. The phenotypes of plants after inoculation with *P. infestans* isolates IPO and H30P04 are indicated.

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Discussion

In this study, we developed the *in vitro* assay as a routine method for monitoring major *R* genes in potato and their corresponding *Avr* gene in *P. infestans*. We observed perfect correlation between the new method and the control detached leaf assay and concluded that the *in vitro* assay can be a new on-hand tool to study the gene-for-gene interaction between potato and *P. infestans*.

Compared to a detached-leaf assay, *in vitro* inoculation has several advantages. First, healthy and uniform *in vitro* plantlets are used, minimizing the influence of the physiological condition of leaves and interference of other pathogens such as *Erysiphe* spp (powdery mildew) in assessment of the resistance of genotypes tested. Second, experiments can be planned more flexibly because of the immediate availability of plant materials and the minimal spaces needed for testing. On the other hand, *in vitro* inoculation also has its limitations. It can not be conveniently used to study the quantitative aspects of potato-*P. infestans* interaction, where quantitative parameters should be used, such as lesion growth rate (LGR) and infection efficiency (IE) (Vleeshouwers et al., 1999). LGR is too difficult to measure on *in vitro* plantlets, although IE scoring would be possible by increasing the number of plantlets. However, in such a scenario, the advantage of fast-testing would be strongly reduced. When plant materials are not available *in vitro*, it is tedious to bring them *in vitro*. For high-throughput phenotyping, this is not a problem since seeds of a segregation population can be sterilized and sown *in vitro*.

The gene-for-gene specificities were genuinely uncovered with the *in vitro* assay, using the standard detached leaf assay as control (Table 2). Both methods gave identical avirulence phenotype of each of the four *P. infestans* isolates tested. We also compared the two methods with potato clones SH and RH on more than ten isolates and found no difference between the two methods (data not shown). Furthermore, we demonsirated that *in vitro* inoculation could sophistically determine the distinct resistance specificities of *R3a* and *R3b* (Table 3). To date, no *R* gene has been found to be detached leaf specific or *in vitro* plantlet specific.

Individual *R* genes respond with their own specific effectiveness (Vleeshouwers et al., 2000). Strong *R* genes, such as *R1* (Stewart and Bradshaw, 2001) always show a full resistance to avirulent *P. infestans* isolates, whereas weak *R* genes, such as *R10* and *R11*, often confer less complete resistance to avirulent isolates (Turkensteen, 1987; Vleeshouwers et al., 2000; Stewart and Bradshaw, 2001; van der Lee et al., 2001). The level of effectiveness of individual *R* genes in the differentials is correlated with their behavior with respect to the four isolates used in the study (Table 2). The weak *R* genes were more difficult to phenotype than the strong ones both on detached leaves and on *in vitro* plantlets. This might account for

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the slight quantitative difference between the two methods on phenotyping *R7*, *R10*, and *R11* as observed in this study.

The reliability of the *in vitro* assay was tested on the *R3* segregating population with two isolates. None of the 126 genotype-isolate combinations displayed a difference between the new method and the control. We concluded that the *in vitro* assay is a reliable alternative for detached leaf tests. It provides a novel high-throughput resistance testing technique of *P. infestans* in potato.

Materials and Methods

Plant material

Plant material used in this study is listed in Table 1. All plant material was maintained on Murashige and Skoog (MS) medium supplemented with 30 g per liter sucrose (MS30) at 24°C and 16 h light/8 h dark. For propagation, single node cuttings were transferred to fresh MS30 medium.

The preparation of plant material for detached leaf assays was previously described (Vleeshouwers et al., 1999). To prepare plantlets for *in vitro* inoculation, fresh shoots were cut and transplanted to glass jars (d=10 cm) or plastic jars (d=15 cm). Each jar contained five cuttings. A two to three cm space was left between plantlet and the inner wall of jars. Plantlets with three to four fully developed leaves were used for *in vitro* inoculation. Depending on the potato genotype, it took two to four weeks from transplanting to inoculation. Generally, it took about six weeks for *in vitro* sown seeds to develop enough leaf material for both DNA isolation and inoculation.

Phytophthora infestans isolates, maintenance and inoculum preparation

P. infestans isolates used in the study are listed in Table 4. The maintenance and preparation of inoculum preparation was performed under sterile conditions, according to previous protocols (Vleeshouwers et al., 1999).

Table 4. *P. infestans* isolates used in this study, their avirulence spectrum and experiment in which they were used. IPO-0 was kindly provided by W. Flier, Plant Research International, The Netherlands, the other isolates were kindly provided by F. Govers, Wageningen University, the Netherlands.

Isolate	Race	Experiment
89148-09	0	
GER8601	1	Avirulence profiling
99018	1.4	Avirulence profiling
91001	7	Avirulence profiling
89148-27	3a.3b.7 [a]	Avirulence profiling
H30P04	3a.7 [a]	*R3* phenotyping
IPO-0	3b [a]	*R3* phenotyping

[a] Huang et al. (2004)

Inoculation and resistance scoring

Detached leaves were spot-inoculated with a zoospore suspension of 5 x 10^4 spores/ml, as previously

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described (Vleeshouwers et al., 1999). For each *in vitro* plantlet, the three largest leaves were inoculated with a zoospore suspension of 2.5×10^4 spores/ml (one spot per leaf) by applying 10 µl droplets on the adaxial side using a continual pipetter (EPPENDORF, Hamburg, Germany). Leaves touching the inner wall or the lid of the jars or the medium were excluded because we found that these leaves were more susceptible than others of the same plants. The inoculated detached leaves and *in vitro* plantlets were incubated in a climate chamber at 15 °C, 16h/8h day/night regime.

At six days postinoculation (dpi), the disease index (DI) was determined on detached leaves by using a five point (1 to 5) scale in which 1 corresponds to spreading lesions with massive sporulation and 5 corresponds to no symptom or dark localized necrosis (HR). The other ratings correspond to intermediate classes of symptoms. Generally, two compound leaves, each five leaflets per leaf, were examined for each isolate-potato genotype combination. The scoring of *in vitro* inoculation was similar to that of a detached-leaf assay except that the unit of observation was *plantlet* instead of *leaflet*. Ten plantlets in two jars, five per jar, were scored on the 1-5 scale for each interaction.

Molecular marker analysis

DNA was extracted from *in vitro* plantlets using a high throughput method (Huang et al., 2004). Two cleaved amplified polymorphic sequence (CAPS) markers, cLET5E4 and GP185, which define a 0.25 cM interval that spans *R3a*, and two amplified fragment length polymorphism (AFLP) markers, PCAMATA_4 and EATCMAGC_15, which define a 0.25 cM interval spanning *R3b*, were used for genotyping the *R3* locus. The PCR conditions were described previously (Huang et al., 2004).

Statistical analysis

Regression coefficient analysis and paired sample T-tests were conducted to examine whether there is a difference between DIs obtained from the detached leaf assay and the *in vitro* assay.

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Chapter 3

The *R3* Resistance to *Phytophthora infestans* in Potato is Conferred by Two Closely Linked *R* Genes with Distinct Specificities

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The *R3* resistance to *Phytophthora infestans* in potato is conferred by two closely linked *R* genes with distinct specificities

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Abstract

The *R3* locus of potato (*Solanum tuberosum* L.) confers full resistance to avirulent isolates of *Phytophthora infestans*, the causal agent of late blight. *R3* resides in the distal part of chromosome 11 and segregates in a potato mapping population, from which a well-saturated amplified fragment length polymorphism (AFLP) map is available. Using a population of 1748 plants, we constructed a high-resolution genetic map at the *R3* locus. Using the combination of fine-mapping and accurate disease testing with specific *P. infestans* isolates, we detected that the *R3* locus is composed of two genes instead of one, with distinct specificities. The two genes, *R3a* and *R3b*, are 0.4 cM apart and have both been introgressed from *S. demissum*, the 'donor' species of most characterized race-specific *R* genes to *P. infestans*. A natural recombinant between *R3a* and *R3b* was discovered in one accession of *S. demissum*. The synteny between the *R3* locus and the tomato *I2* locus is discussed.

Introduction

The central dogma of molecular plant pathology is the gene-for-gene model, which states that, for every dominant resistance (*R*) gene in the plant, there is a corresponding dominant avirulence (*Avr*) gene in the pathogen (Flor, 1971). The presence of matching gene pairs controls the outcome of the interaction of many pathosystems (Thompson and Burdon, 1992), including the interaction between potato (*Solanum tuberosum* L.) and its oomycete pathogen *Phytophthora infestans* (Black et al., 1953; van der Lee et al., 2001). A corollary of the gene-for-gene model is that receptors encoded by *R* genes can directly or indirectly perceive the products of corresponding *Avr* genes, enabling recognition of the pathogen and subsequent elicitation of an array of plant defense responses that eventually lead to resistance (Keen, 1990; Dangl and Jones, 2001). In many cases, the induced defense is

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manifested as a hypersensitive response (HR) which is associated to a programmed cell death at the initial site of infection (Morel and Dangl, 1997). Unexceptionally, HR was found to be associated with all forms of resistance to *P. infestans*, including race or cultivar-specific resistance, partial resistance, and nonhost resistance, indicating that recognition occurs independently of the type of resistance (Kamoun et al., 1999; Vleeshouwers et al., 2000). To understand the mechanisms underlying recognition, it is essential to isolate the interacting gene pairs from both the host and the pathogen. For this purpose, single dominant *R* genes and *Avr* genes are preferred because they are genetically more accessible.

Late blight, caused by *P. infestans*, is the most devastating disease for potato production worldwide (Fry and Goodwin, 1997). Resistance conferred by dominant *R* genes is easier to manipulate than quantitative resistance. A number of *R* genes for late blight resistance have been introgressed from wild relatives into cultivated potato through sexual and somatic hybridization. Some of these have been mapped to the potato genome through linkage to specific DNA markers (Gebhardt and Valkonen, 2001). Recently, two of them have been cloned. One is *R1*, a race-specific *R* gene originating from *S. demissum* (Ballvora et al., 2002). Another is *RB* or *Rpi-blb1*, which confers broad-spectrum resistance and is derived from *S. bulbocastanum* (Song et al., 2003; van der Vossen et al., 2003). Both genes are members of the *R* gene class predicted to encode receptors with coiled coil (CC), nucleotide binding site (NBS), and leucine-rich repeat (LRR) domains (Ballvora et al., 2002; Song et al., 2003; van der Vossen et al., 2003).

Previously, *R3* was mapped as a single dominant locus on the distal part of the short arm of potato chromosome 11, closely linked to restriction fragment length polymorphism (RFLP) markers GP250A, GP185, and TG105A (El Kharbotly et al., 1994). The short arm of chromosome 11 in potato is syntenic to the long arm of chromosome 11 in tomato (Tanksley et al., 1992; Dong et al., 2000). Like many other *R* loci, *R3* resides in a hot spot for resistance to various pathogens. A number of major *R* loci and quantitative trait loci (QTL) for resistance from Solanaceae crops have also been anchored in the same region (Grube et al., 2000; Pan et al., 2000), including another two *P. infestans* resistance genes, *R6* and *R7* (El Kharbotly et al., 1996). To date, the tomato *I2* gene that confers resistance to *Fusarium* is the only *R* gene cloned from this region (Ori et al., 1997a; Simons et al., 1998).

In this report we describe the construction of a high-resolution genetic map of the *R3* locus using the saturation from the ultra-high dense potato map with over 10,000 amplified fragment length polymorphism (AFLP) markers (http://www.dpw.wageningen-ur.nl/uhd) and the resolution from a large segregating F1 population. Interestingly, two functional *R* genes instead of one were found at the

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locus and they displayed a differential reaction to some well-defined *P. infestans* isolates. The occurrence of two *R* genes at the *R3* locus was confirmed in a natural population of *S. demissum*. The colinearity between tomato and potato in this region of chromosome 11 enabled the development of *R3*-flanking markers from tomato sequences and the association of the locus with the *I2* locus of tomato.

Table 1. Plant materials used in this study.

Clone	Abbr.	Species	Known *R* Gene	Remark
RH89-039-16	RH	*S. tuberosum*	None	Paternal clone of the RH x SH *R3* mapping population
SH83-92-488	SH	*S. tuberosum*	*R3*	Maternal clone of the RH x SH *R3* mapping population
Esc.42	Esc	*S. tuberosum*	*R1, R3*	Paternal clone of the J91-6164 *R3* mapping population [a]; introgression study
84-1031-29	1031	*S. tuberosum*	None	Maternal clone of the J91-6164 6164 *R3* mapping population; introgression study
J91-6164-21	6164	*S. tuberosum*	*R3*	A progeny of J91-6164; introgression study
Reasal	Reasal	*S. tuberosum*	*R1, R3*	Common ancestor of Esc and SH; syn. to 4788-15 [b]; introgression study
Mastenbroek *R3*	Ma*R3*	*S. tuberosum*	*R3*	The *R3* differential developed by Mastenbroek; syn. to CEBECO 4642-1 [c]
1024-2	1024	*S. tuberosum*	None	Introgression Study
CE-10	CE	*S. tuberosum*	None	Introgression Study
Katahdin	KA	*S. tuberosum*	None	Introgression Study
CGN17810	17810	*S. demissum*	*R1, R2, R3, R4, R7, R8*	One of the *R3* donor accessions; introgression study
CGN18313	18313	*S. demissum*	None [d]	Introgression study

[a] El Kharbotly et al. 1994
[b] Mastenbroek 1953
[c] van der Lee et al. 2001
[d] this study revealed that 18313 carries *R3b* (Fig. 4).

Results

Identification and localization of *R3* in the ultra-high dense map

R3 was previously mapped on the distal part of chromosome 11 by using a diploid segregating population, J91-6164, which was derived from the cross between Esc.42 (Esc, Table 1, *R1r1, R3r3*) and 84-1031-29 (1031, Table 1, *r1r1, r3r3*). Esc is a dihaploid derivative from potato cv. Escort (El Kharbotly et al., 1994). SH83-92-488 (SH, Table 1), the maternal clone of the mapping population of the ultra-high dense map (http://www.dpw.wageningen-ur.nl/uhd), shares its genetic background with

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Escort. Pedigree information revealed that both SH and Escort descended from potato cv. Reaal that carries *R1* and *R3* (Table 1, Mastenbroek 1953).

Table 2. PCR markers used in this study and listed according to their position on the high-resolution map. Their Bin position, phase to *R3* (C for coupling and R for repulsion), primers, approximate size of amplified fragment on genomic DNA of SH, and restriction enzymes to detect polymorphism are indicated.

Marker	Bin	Phase	Primer (5'-3')	Tm (°C)	Fragment size (bp)	Restriction enzyme
St3.3.11	46	C	GCT AAG CTG GGA TTG TTG TCT TAC TGT GCC ACC CGT TGA G	52	380	a.s. [b]
cLET5E4	65/66	C	CCA GGC ATG CTC AAT TTG GAG T TTC CCT GTT TGG ACT ACT TGT GGA	55	310	*Hha*I
St1.1	65/66	C	GCT CAT TCG ACT TAA AGG TTG TTG GGC AGC TCC CAT ATT TCA CTT CTC	60-52 [a]	450	*Hae*III
TG105	65/66	C	TCA CAT GAG CTG GGA GAA AT AAA GGC CTG TTG CTG AGA G	54	650	*Hin*fI
GP250	65/66	C	ACC AGT AGG ACC ACC ACC AAC AAT GAT CGT GAC GGC TCT ACT CTT TTA TGA	60-62 [a]	410	*Vsp*I
STM0025	65/66	C	GTT CAT GAT TGT GAA TGC TC ATG ACT CAA CCC CAA ATG	59	170	a.s. [b]
GP185	65/66	C	CTG GTA ATA GTA GTA ATG ATT CTT CGT C TTG TTC AAT GGA GCA CTT GC	54	440	*Bst*UI
TG28	65/66	C	GAG AGG GGA CAC TTT TAT TTA TTC A GAG ATC TTC CCG CCG CTG TG	49	1600	a.s. [b]
CT129	67	R	CGA GGG GGC GAA GGA TT CCA TGA GAT AAA CGA GGA ACC AGT	52	360	*Tsp*509I

[a] touchdown PCR with Tm gradually decreasing from 60 °C to 52 °C
[b] allele specific.

To verify whether this genetic background includes *R3* so as to enable the utilization of the global coverage with AFLP markers on the ultra-high dense map, we analyzed the resistant clones SH, J91-6164-21 (6164, Table 1), Reaal, and susceptible clones RH89-039-16 (RH, Table 1) and 1031 with PCR markers GP250, TG105 and STM0025 (Table 2). The clone 6164 is a progeny of the previous mapping population J91-6164 (El Kharbotly et al. 1994). It carries only *R3* but not

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R1. RH is the paternal clone of the population of the ultra-high dense map. In J91-6164, GP250 and TG105 mapped as RFLP markers are proximal to *R3* (El Kharbotly et al. 1994). STM0025 is 5 cM distal to GP250 (Milbourne et al., 1998). The band pattern of all the three PCR markers suggested that at the *R3* region all the resistant clones carry a genomic fragment, which is identical-by-descend (Fig. 1A).

To confirm the presence of *R3* in SH, we characterized SH, 6164, RH, and 1031 for race-specific resistance with a panel of *P. infestans* isolates (Table 3) on both detached leaves and *in vitro* plantlets. SH and 6164 displayed a typical hypersensitive response when inoculated with *P. infestans* isolates 89149-9, IPO-0, and H30P04 (Table 3), which were all avirulent to the *R3* differential, Mastenbroek *R3* (Ma*R3*, Table 1, van der Lee et al. 2001). Both SH and 6164 exhibited full susceptibility to *P. infestans* isolates 89148-27 and 90128 (Table 3), which are both virulent on Ma*R3*. Control clones RH and 1031 were susceptible to all the five isolates (Fig. 1B). This demonstrated that SH carries an *R* gene (genes) with the same specificity as *R3*.

Table 3. *P. infestans* isolates used in this study and their virulence profiles before and after this study.

Isolate	Virulence profile		Source
	Before this study	After this study	
89148-9	0	0	F. Govers, Wageningen University, The Netherlands
IPO-0	0	3b	W. Flier, Plant Research International, The Netherlands
H30P04	?	3a.7	
89148-27	3.7	3a.3b.7	F. Govers, Wageningen University, The Netherlands
90128	1.3.4.6.7.8.10.11	1.3a.3b.4.6.7.8.10.11	

To test whether the *P. infestans* resistance in SH is inherited as a single *R* gene, we examined the segregation for resistance in the mapping population (SH x RH) of the ultra-high dense map (http://www.dpw.wageningen-ur.nl/uhd). We inoculated the population with *P. infestans* isolate IPO-0. The distinction between the resistant and the susceptible phenotypes was clear, i.e., the resistant individuals showed a dark localized necrosis (HR) at three to four days post-inoculation (dpi) while the susceptible ones gave extensive sporulation at five dpi. This suggested the resistance was controlled by major gene(s). In a total of 108 individuals tested, 46 were resistant and 62 were susceptible, which fitted the Mendelian 1:1 ratio. Although the segregation slightly skewed toward susceptibility, the ratio supported the inheritance

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of a single dominant *R* gene ($\chi^2 = 2.37$; $p \geq 0.1$).

Subsequently, the *R3* locus was mapped to the distal part of the short arm of chromosome 11 on the ultra-high dense AFLP map, confirming the previous map position in J91-6164 (El Kharbotly et al., 1994). Due to the limited population size (n=130), the over 10,000 AFLP markers cannot be localized individually. Instead of "centi Morgan" (cM), the genetic distance unit for the ultra-high dense map is "Bin". A Bin is defined as a genetic interval flanked by a recombination event on either side. All markers within a Bin are cosegregating. The length of a Bin is a genetic interval of ~0.8 cM (=1/130). *R3* was mapped in either Bin SH11B65 (65) or 66. Due to the loss of the diagnostic plant that could separate these two neighboring Bins, we can no longer precisely place *R3* to one of the two Bins. *R3* therefore cosegregates with all the markers mapped in the combined Bin 65/66, i.e., GP250, TG105, STM0025, and 43 cosegregating AFLPs. Bin 67 is the most distal Bin of the short arm of chromosome 11. Based on pedigree information, marker analysis, inoculation results and genetic mapping, we conclude that SH carries *R3*.

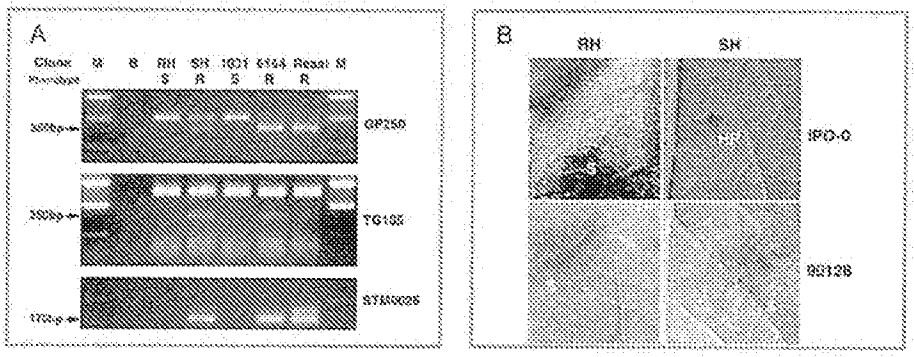


Fig. 1. Molecular and biological evidence for the presence of *R3* in SH.
A. Analysis of the genomic DNA from RH, SH, 1031, 6164, and Reaal with markers GP250 (*Vsp*I), TG105 (*Hin*fI), and STM0025. The phenotype (R-resistant, S-susceptible) of these clones inoculated with *P. infestans* isolate IPO-0 is indicated. The sizes of polymorphic bands are indicated on the left. DNA size markers are indicated as 'M' and blank control as 'B'.
B. A gene-for-gene interaction of potato and *P. infestans*. RH and SH leaves inoculated with isolates IPO-0 (race 0) and 90128 (race 1.3.4.6.7.8.10.11), five dpi. A race-specific resistance response occurred on SH leaves upon inoculation with IPO-0 (HR). Extensive sporulation (S) was noted in the other three compatible interactions.

Screening for informative recombination at the *R3* locus

For high-resolution mapping of *R3*, it was essential to define the order of those cosegregating markers with meiotic cross-overs. We applied a flanking marker strategy to screen for recombination events around the *R3* locus (Tanksley et al., 1995). Instead of laborious analysis with AFLP markers available from flanking

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Bins, we took advantage of the colinearity between tomato and potato at the *R3* region of chromosome 11 (Tanksley et al., 1992; Dong et al., 2000) and the sequences of known RFLP markers from the two species to develop easy-scoring PCR markers.

Based on their position, markers cLET5E4, TG26, CT120 from tomato and St3.3.11, St1.1, and GP185 from potato were chosen for PCR marker development (Table 2). St3.3.11 was mapped in Bin 46, about 16 cM proximal to the *R3* locus. Like GP250 and TG105, cLET5E4, GP185, St1.1, and TG26 co-localized with *R3* in the ultra high dense map and then were mapped in Bin 65/66. After testing with 250 additional SH x RH plants, we mapped the marker cLET5E4 proximal to the *R3* locus with three recombinants between the marker and the locus. CT120 was placed in Bin 67 and in repulsion to *R3*. It served as a distal flanking marker. Therefore, the cLET5E4-CT120 interval spans a genomic region with the *R3* locus in the center. We used these two markers for recombinant screening of 1748 SHxRH progeny (Fig. 2). Out of these, we identified 34 recombinants in the cLET5E4-CT120 interval.

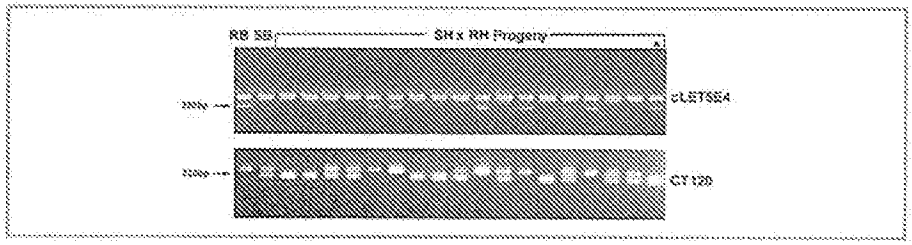


Fig. 2. Screening for recombination events near the *R3* locus in the SH x RH progeny with two flanking markers, cLET5E4 (*Hha*I) and CT120 (*Tsp*509I). The sizes of the diagnostic bands are indicated on the left. RB, resistant bulk consisting of 20 resistant plants; SB, susceptible bulk consisting of 20 susceptible plants. The presence of the 220 bp band of cLET5E4 is associated with the absence of the 320 bp band of CT120 in all progeny, except in the recombinants. The right lane (*) shows a recombinant pattern.

Detection of two *R* genes at the *R3* locus

The resistance phenotype of the 34 recombinants was determined with the *P. infestans* isolates 89148-9, H30P04, and IPO-0, which all have an incompatible interaction with SH. Twenty-six recombinants were either resistant or susceptible to all the three isolates. Surprisingly, we found the remaining eight recombinants differed in their response to H30P04 and IPO-0. Six of these, SW8536-18, SW8537-033, SW8539-004, SW8540-025, SW8540-054, and SW8540-309, were resistant to *P. infestans* isolate IPO-0 and susceptible to isolate H30P04, while the other two recombinants, SHRHC8-#130 and SW8540-325, were just the opposite,

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i.e., susceptible to IPO-0 and resistant to H30P04. All these eight recombinants were resistant to 89148-9. The differential reaction to the isolates was confirmed with repeated inoculations using detached leaves and *in vitro* grown plantlets (Fig. 3).

The recombinant SHRHC8-#130 is from the mapping population RH x SH of the ultra-high dense map and it has a cross-over in the cLET5E4-CT120 interval. To exclude the possibility of a second locus that is not within the interval and affects the resistance to *P. infestans* isolate H30P04, we inoculated the mapping population RH x SH with H30P04 as we previously did with isolate IPO-0. None of the 108 plants tested but SHRHC08-#130 displayed a differential reaction to these two isolates. Genetic mapping revealed that resistance to isolate H30P04 in the population was inherited as a single *R* gene and was mapped within the cLET5E4-CT120 interval. No additional genetic component outside the interval affected the phenotype.

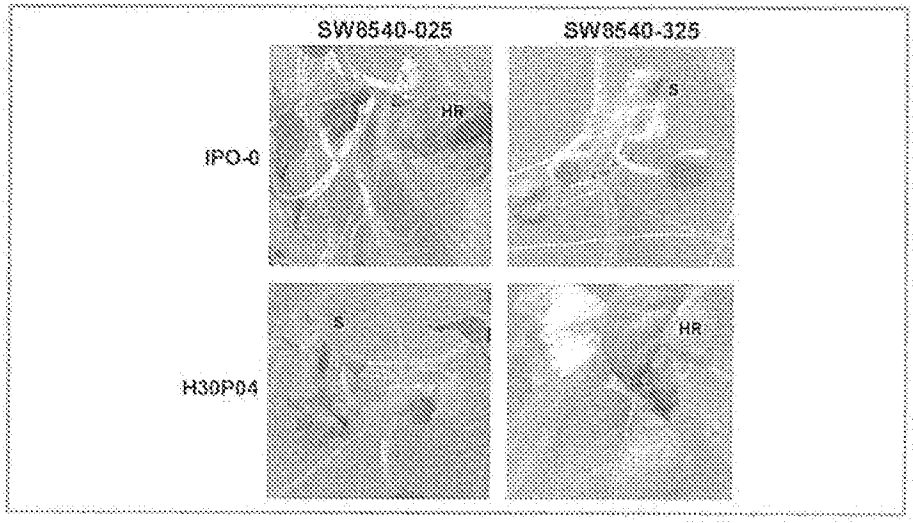


Fig. 3. Phenotype of differential resistance response at 6 dpi with *P. infestans* isolates IPO-0 and H30P04 on *in vitro* plants. SW8540-025 is resistant (HR) to IPO-0 and is susceptible to H30P04 as shown by the sporulating lesions (S). SW8540-325 shows the opposite response.

All the 34 recombinants in the cLET5E4-CT120 interval were subjected to characterization with all molecular markers from Bin65/66 and Bin67. The eight recombinants that showed a differential response to *P. infestans* isolates IPO-0 and H30P04 were found to carry a cross-over in the GP250-EATCMAGC_15 interval, which is a sub-interval of the cLET5E4-CT120 interval (Fig. 4AB). The remaining 26 recombinants that were either resistant or susceptible to both isolates did not have a cross-over in the sub-interval. The marker pattern of the 34 recombinants indicated that the difference in isolate specificity was due to cross-overs between two closely linked *R* genes with distinct resistance spectra.

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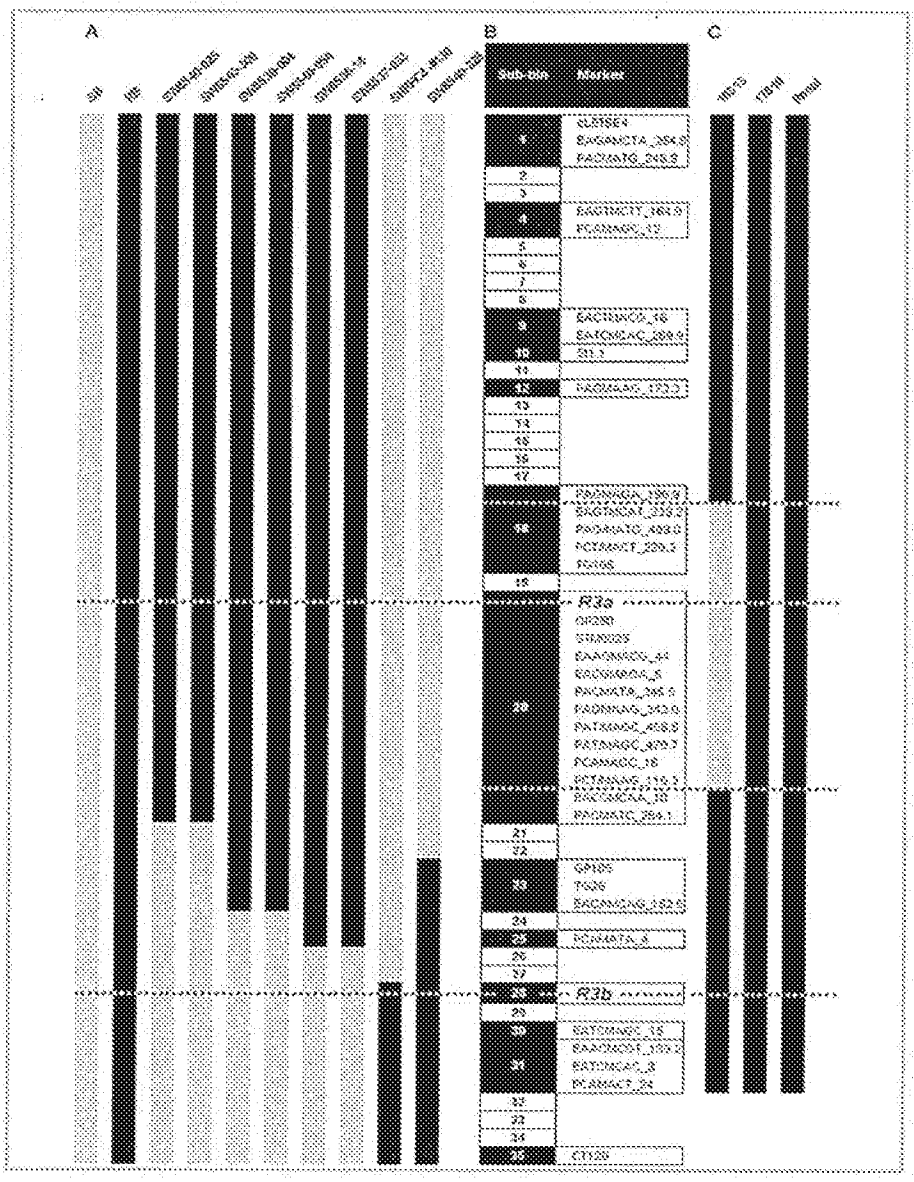


Fig. 4. High-resolution genetic map of *R3a* and *R3b*.

A. Graphical genotypes of the susceptible bulk (SB, grey bar), the resistant bulk (RB, black bar), and the eight SH x RH progeny which displayed a differential response to H30P04 and IPO-0. The transition of black and grey indicates a cross-over.

B. The high resolution map of the *R3* locus. The Sub-bins are numbered from one to 35 in a proximal-distal order. The Sub-bins filled with marker(s) and/or gene are shown with black background and white font. The dotted lines indicate the positions of *R3a* and *R3b* on the genotypes listed in A and C. Graphical genotypes of Reaal and two *S. demissum* accessions, 18313 and 17810. Reaal and 17810 are identical with RB. Two dotted lines, splitting Sub-bin 18 and 20, indicate the interval of susceptible "island" at 18313. Marker CT120 was not investigated in the three clones because it is a repulsion phase marker.

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The data on isolate specificities, mapping, and marker pattern clearly demonstrated that the phenotype of *R3* resistance in SH is conferred by two closely linked *R* genes. Hereby we designate them as *R3a* and *R3b*. *R3a* confers resistance to isolate IPO-0 and not to H30P04, *R3b vice versa*. Both genes confer resistance to isolate 89148-9. Associated with the nomination of *R3a* and *R3b*, we can now designate IPO-0 as race 3b and H30P04 as race 3a.7. The isolate 89148-9 is still race 0 in this scenario (Table 3).

Construction of a high-resolution map at the *R3* locus

The 34 recombinants identified from the cLET5E4-CT120 interval from the 1748 SH x RH plants divided the interval into 35 Sub-bins (Fig. 4B). The genetic length of a Sub-bin is equivalent to ~0.05 cM (=1/1748). The resolution achieved with the high-resolution map of the *R3* locus is 16 times higher than that of the ultra-high dense map (0.05 cM versus 0.8 cM). A total of 35 molecular markers (27 AFLP markers and eight PCR markers) from the interval were assigned to the Sub-bins. The distribution of cross-overs and markers was non-random within the cLET5E4-CT120 interval. Nearly one third of the markers were clustered in Sub-bin 20, while no marker was placed in 22 of the 35 Sub-bins (Fig. 4B).

The *R3a* gene resides in Sub-bin 20, cosegregating with GP250, STM0025 and ten AFLPs. TG105 and four AFLP markers were the closest proximal markers, separated from *R3a* by two recombination events. GP185, TG26 and EACAMCAG_152.6 were the closest distal markers, separated from *R3a* by three recombination events (Fig. 4B). The order of TG105-GP250-GP185 in SH was well consistent with the previous mapping result (Gebhardt et al., 1994). Twenty markers clustered in the TG105-GP185 interval that consists of six Sub-bins.

The *R3b* gene was mapped in Sub-bin 28, eight Sub-bins (~0.4 cM) distal to *R3a* (Fig. 4B). No marker cosegregates with the gene. The AFLP markers PCAMATA_4 and EATCMAGC_15 are its closest markers, separated by three and two recombinations, respectively. The PCAMATA_4-EATCMAGC_15 interval for *R3b* spans the same genetic distance as the TG105-GP185 interval of *R3a*, i.e., six Sub-bins, but contains much less markers (two versus 20).

A natural recombination between *R3a* and *R3b* in *Solanum demissum*

Introgression analysis based on pedigree information can provide additional resolution to determine the order of markers and gene of interest, thus it can serve as a supplemental tool to fine mapping based on segregating population (Kanyuka et al., 1999). Reaal is one of the best-characterized clones that carry the *R3* locus introgressed from *S. demissum* (Mastenbroek, 1953). There are five generations of

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crossing and one round of dihaploidization between Reaal and SH (data not shown). The introgression study with markers tightly linked to *R3a* (GP250, STM0025, and TG105) between SH and Reaal suggested that *R3a* in SH descended from Reaal (Fig. 1A). However, these data cannot exclude the possibility that *R3b* in SH was derived from another clone rather than Reaal and *S. demissum*.

To check the origin of *R3b* and confirm the SH-Reaal link on *R3a*, we conducted a fine comparison of SH, Reaal, two *S. demissum* accessions (17810 and 18313, Table 1), and four susceptible *S. tuberosum* clones (1024, 1031, CE, and KA, Table 1) with all the 29 coupling phase markers in the cLET5E4-CT120 interval. SH, Reaal and 17810 showed identical marker patterns in the interval (Fig. 4A and 4C). Notably, 17810 is synonymous to CPC2127, one of the *R3* donor accessions of *S. demissum* (Black, 1950). This similarity was not found for any of the four susceptible clones (1024, 1031, CE, and KA). Their marker pattern at the interval was almost identical to that of RH, the susceptible parental line. In the 29 coupling phase markers analyzed, only one marker pattern differed between 1031, CE, KA, and RH and four differed between 1024 and RH. Together with pedigree information, this result strongly supports the notion that the *R3a-R3b*-carrying chromosome region in SH was descended from Reaal and originated from *S. demissum*.

Interestingly, marker analysis revealed that another *S. demissum* accession, 18313, contains an "island" of susceptible alleles from Sub-bin 18 to 20 flanked by two blocks of resistance alleles (Fig. 4B and 4C). The clustering of markers in the "island" was confirmed by physical mapping (data not shown). The "island" in 18313 may offer a further resolution for determination of marker order relative to *R3a* because Sub-bin 20 can be divided into two parts based on the result. The AFLP markers EACGMCAA_10 and PACMATC_264.1 were separated from the other markers in the Sub-bin 20 (Fig. 4B and 4C). From the marker pattern, 18313 was predicted to carry *R3b*.

The accession 18313 was subsequently tested for resistance specificity with *P. infestans* isolates 89148-9, IPO-0, and H30P04. It did display a differential reaction to these isolates that was the same as the reaction of the recombinants SHRHC8-#130 and SW8540-325, i.e., resistant to 89148-9 and H30P04 but susceptible to IPO-0. Therefore, we conclude that 18313 does carry *R3b* but not *R3a* and is a natural recombinant of these two *R* genes. The characterization of this accession not only confirmed the presence of the two functionally distinct genes at the *R3* locus, but also further delimited *R3a* to the upper part of the otherwise intact Sub-bin20 (Fig. 4B and 4C). Thus the introgression analysis resulted in additional resolution instrumental for fine mapping the *R3* locus.

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Discussion

In this report we describe the construction of a high-resolution genetic map reaching a 0.05 cM resolution at the *R3* locus and the finding that the locus consists of two distinctly functional *R* genes, *R3a* and *R3b*. The high resolution together with accurate disease testing with specific *P. infestans* isolates enabled the detection of two closely linked *R* genes with different resistance spectra. The introgression analysis confirmed that both genes in SH were derived from *S. demissum* and provided a further resolution to narrow down the interval for the *R3a* gene. The resolution obtained from both recombination analysis and introgression study will assist the isolation of both genes using a positional cloning strategy.

For high-resolution genetic mapping, two aspects are essential, marker saturation, i.e., the amount of sequence polymorphisms in the target region, and recombination frequency, i.e., the amount of meiotic cross-overs to resolve the markers. However, these two factors are often negatively correlated. Recombination in general is inhibited by sequence heterogeneity (Borts and Haber, 1989; Metzenberg et al., 1991; Dooner and Martínez-Ferez, 1997). The frequency of mitotic recombination in yeast can be affected noticeably by less than 1% nucleotide sequence divergence (Datta et al., 1997). Recombination frequency is about eight-fold higher in the *Mi* region of a *Lycopersicon peruvianum* cross than a *Lycopersicon esculentum* cross with *Mi*-introgression from *L. peruvianum* (Kaloshian et al., 1998). This is also the case for the *R3* locus in SH where we found a significant association between sequence heterogeneity, measured by the number of markers per cM and the reduction of recombination frequency using the tomato map as reference (Tanksley et al., 1992; Fulton et al., 2002). The cLET5E4-TG105, TG105-TG26, and TG26-CT120 intervals in SH have 16.5, 80, and 15 markers per cM, respectively. This is correlated to 9, 16, and 4 times less recombination, respectively (r=0.918). *R3b* resides in a region with higher recombination frequency but less saturation than *R3a*.

Most of the *R* genes that have been characterized at the molecular level belong to complex loci with tightly linked paralogs. Large arrays of similar sequences allow for equal or unequal recombination events, resulting in the formation of new gene family members (Michelmore and Meyers, 1998; Ellis et al., 2000; Hulbert et al., 2001). However, the evolutionary fate of new members in complex resistance loci may largely depend on the interaction between plants and their microbial environment. The majority of complex resistance loci carry a functional *R* gene and multiple paralogs with no detectable function, although they are not obvious pseudogenes and appear capable of coding for proteins similar to the functional *R* genes (Hulbert et al., 2001). Only a few complex resistance loci

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comprise more than one detectable functional *R* gene (Dixon et al., 1996; Parniske et al., 1997; Botella et al., 1998; Wang et al., 1998; Takken et al., 1999; van der Vossen et al., 2000). In contrast to the *R1* and *R8 or Rpi-blb1* loci where only one functional gene for *P. infestans* resistance was found in each locus (Ballvora et al., 2002; Song et al., 2003; van der Vossen et al., 2003), the *R3* locus harbors two *R* genes with distinct resistance specificities to the oomycete pathogen.

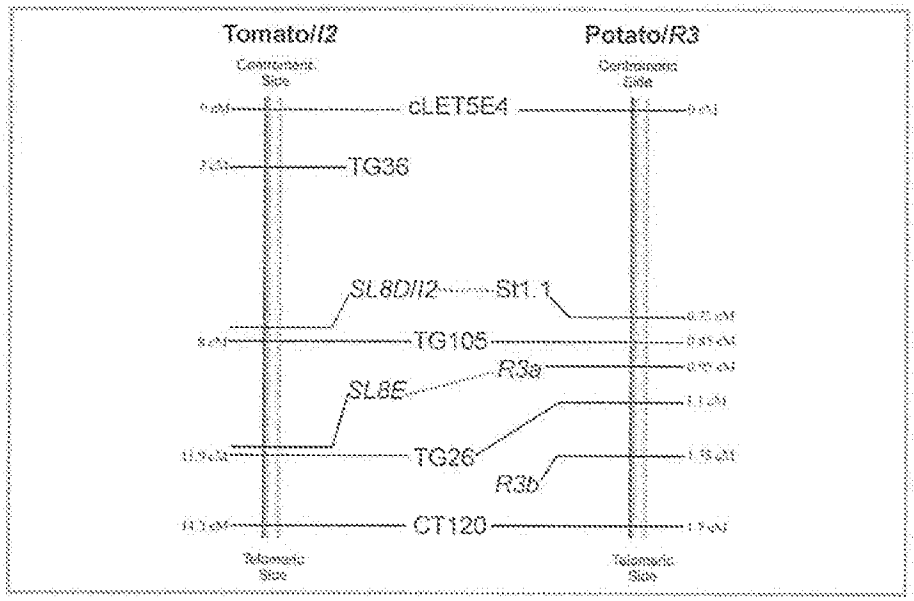


Fig 5. The synteny between the *R3* locus on the short arm of chromosome 11 in potato clone SH and the *I2* locus on the long arm of chromosome 11 of tomato. The left and right solid bars represent the tomato and potato genome, respectively. Mapped markers or genes are connected by black lines. On the tomato map, the positions of TG36, TG105, TG26, and CT120 were according to Tanksley et al. (1992), cLET5E4 to Fulton et al. (2002) and to the Solanaceae Genomics Network (http://www.sgn.cornell.edu), and *SL8D/I2* and *SL8E* to Ori et al. (1997). The positions of markers and genes on the potato map were determined in this study. For the convenience of comparison, the 'Sub-bin' distances in potato (Fig. 4B) have been changed into the 'cM' (1 Sub-bin≈0.05 cM). The dotted line indicated the putative relation between *SL8D* and St1.1, and *SL8E* and *R3a*, respectively.

Map positions of resistance genes appear to be well conserved in the Solanaceae (Grube et al., 2000; Pan et al., 2000). The *R3* locus was mapped on the short arm of chromosome 11 in potato (syntenic to the long arm of chromosome 11 in tomato) where potato and tomato show a high macro-colinearity (Tanksley et al., 1992). Up to date, the *SL8* (*I2*) gene family in tomato was the only *R* gene family that has been molecularly characterized on this chromosome arm. This gene family distributes to five genomic positions in tomato and three of these, *SL8C*, *SL8D* and

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SL8E (in order from the centromere to telomere), were mapped to this chromosome arm (Ori et al., 1997b; Simons et al., 1998). The *SL8C* cluster maps within 10 cM of the *Stemphylium* spp. *R* gene *Sm* (Behare et al., 1991). The *SL8D* cluster lies between TG36 and TG105 and contains *I2* and six paralogs (Ori et al., 1997b; Simons et al., 1998). The marker St1.1 has 91% nucleotide homology with the NBS region of *I2* and localizes between cLET5E4 and TG105. Therefore, St1.1 may be regarded as one of the potato orthologs of the *SL8D* locus (Fig. 5). In tomato, the *SL8E* locus resides between TG105 and TG26 (0.25 cM to TG26) and consists of only one paralog, as indicated by Southern blot analysis (Ori et al., 1997b). In this study, *R3a* was also mapped in the TG105-TG26 interval, possibly resembling the *SL8E* locus in tomato. *R3b* is distal to TG26 (Fig. 4B and 5). Moreover, we found several *I2*GAs (tomato *I2* resistance gene analogs) cosegregating with *R3a* and *R3b* (data not shown), but the relationship of these *I2*GAs with *R3a* and *R3b* is yet to be determined. At the *SL8E*-syntenic region of *S. demissum*, *I2*GAs not only grew in number, but also duplicated over TG26. It will be interesting to investigate whether this expansion is associated with the capacity to recognize additional elicitors from *P. infestans*.

The genomic region of *R3* is rich in functional diversity for *P. infestans* resistance. Besides *R3*, two additional haplotypes, *R6* and *R7*, confer distinct specificities (El-Kharbotly et al. 1996). The *R3* locus might have passed through multiple rounds of gene duplication and diversifying selection to produce new specificities for *P. infestans* resistance and thus, could be a good example to study the dynamic evolution of the potato genome in the perspective of co-evolution with this (in)famous oomycete pathogen.

Materials and Methods

Plant materials

Plant materials used in the study and their resistance profiles are listed in Table 1. The two *S. demissum* accessions were kindly provided by the Centre for Genetic Resources of the Netherlands (Wageningen). All other plant materials were from the Laboratory of Plant Breeding. The population segregating for *R3* was derived from a diploid cross between SH as maternal clone and RH as paternal one. From this cross, 130 progeny have been used for constructing the ultra-high dense map (http://www.dpw.wageningen-ur.nl/uhd). For high-resolution mapping, 1618 additional plants from the same cross were used. In total, 1748 individuals were used to search for recombination events occurring near *R3*. For introgression study, five plants from each *S. demissum* accession were used for inoculation. From each accession, DNA pooled from the five individual plants was subjected for marker analysis.

P. infestans isolates and disease test

P. infestans isolates used in the study and their virulence profiles are listed in Table 3. Their virulence profiles were confirmed using a standard differential set of potato clones (van der Lee et al. 2001). Inoculum preparation was performed as described previously (Vleeshouwers et al., 1999). Resistance

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testing was performed on detached leaves (Vleeshouwers et al., 1999) and/or on *in vitro* plantlets (Huang et al., manuscript in preparation). Resistance scoring was performed as described by van der Lee et al. (2001).

DNA isolation

Genomic DNA from plants grown in greenhouse was isolated according to van der Beek et al. (1992). For DNA extraction of *in vitro* seedlings, the above method was modified to a high-throughput procedure using the Retsch machine (RETSCH INC., Haan, Germany) and 96-deep-well COSTAR microtiterplates (CORNING INC., New York).

Molecular markers

PCR markers were derived from RFLP/SSR markers which have been previously mapped on chromosome 11 of potato (GP185, GP250, St1.1, St3.3.11, and STM0025) and tomato (cLET3E4, CT120, TG26, and TG105) (Tanksley et al., 1992; Gebhardt et al., 1994; Leister et al., 1996; Milbourne et al., 1998). An overview of the markers is given in Table 2. The sequence of GP185 was kindly provided by Dr. Barbara Baker, University of California at Berkeley. Other sequences were obtained from the NCBI database or from the Solanaceae Genomics Network (http://www.sgn.cornell.edu) or from our own sequencing project. Primer pairs were designed with the PrimerSelect module of the DNASTAR package (DNASTAR INC., Madison, Wisconsin). For PCR analysis, 15 µl reaction mixtures were prepared containing 20 ng DNA, 7.5 ng of each primer, 0.1 mM of each dNTP, 0.3 units Taq-polymerase (Supertaq, Enzyme Technologies Ltd, UK), 10 mM Tris-HCl pH 9, 1.5 mM $MgCl_2$, 50 mM KCl, 0.1% Triton X-100 and 0.01% (w/v) gelatin. The PCRs were performed for 35 cycles using the following profile: 30 seconds DNA denaturation at 94°C, 30 seconds annealing at Tm (Table 2) and 45 seconds elongation at 72°C. As a first step in PCR-amplification DNA was denatured for five min at 94°C and finalized by an extra seven min elongation step at 72°C. The amplification reactions were performed in a Biometra T-Gradient thermocycler (WESTBURG, Leusden, The Netherlands). Depending on the marker, the PCR product was digested with an appropriate restriction enzyme (Table 2). Subsequently, the (digested) PCR products were analyzed by electrophoresis in agarose gels.

AFLP analysis (Vos et al., 1995) was performed on a Licor sequencer (LI-COR, Lincoln Nebraska) using fluorescent-labeled *Eco*RI or *Pst*I primers.

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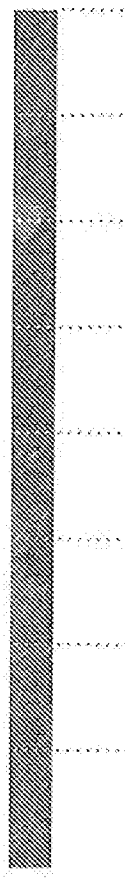
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Chapter 4

Comparative Genomics Enables the Isolation of the *R3a* Late Blight Resistance Gene in Potato

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Comparative genomics enabled the isolation of the *R3a* late blight resistance gene in potato

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Abstract

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. 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. In this report, we adopted a local RGA approach using genomic information from the model Solanaceous plant tomato to isolate *R3a*, a potato gene that confers race-specific resistance to the late blight pathogen *Phytophthora infestans*. *R3a* is a member of the *R3* complex locus on chromosome 11. Comparative analyses of the *R3* complex locus with the corresponding *I2* complex locus in tomato suggest that this is an ancient locus involved in plant innate immunity against oomycete and fungal pathogens. However, the *R3* complex locus has evolved after divergence from tomato and the locus has experienced a significant expansion in potato without disruption of the flanking colinearity. This expansion has resulted in an increase in the number of *R* genes and in functional diversification, which has probably been driven by the co-evolutionary history between *P. infestans* and its host potato. Constitutive expression was observed for the *R3a* gene, as well as some of its paralogues whose functions remain unknown.

Introduction

Comparative genomics investigates the similarity and differences in structure and function of genomes across taxa. Full genome sequences of Arabidopsis (AGI, 2000) and rice (Goff *et al.*, 2002; Yu *et al.*, 2002) and several other ongoing sequencing projects will offer unprecedented resources to study the evolution of sequence and function of orthologous genes and to understand diversification and adaptation. A fundamental and practical question in comparative genomics is whether this vast amount of sequence information from model plant species will facilitate the cloning

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of genes with agronomic importance from crop species with larger genomes. Resistance to plant pathogens, often defined by single dominant disease resistance (*R*) genes (Dangl and Jones, 2001), is an important crop trait that could benefit from the sequencing of model species. This benefit, however, will largely depend on the given plant family. Extensive loss of colinearity has been reported within crucifers and grasses (Gale and Devos, 1998; Paterson *et al.*, 2000). Comparative analysis based on DNA sequences has revealed that disease resistance (*R*) loci may be evolving faster than the rest of the crucifer and grass genomes (Gale and Devos, 1998; Leister *et al.*, 1998; Paterson *et al.*, 2000). In contrast, the remarkable conservation of gene order (Bonierbale *et al.*, 1988) makes the Solanaceae, a botanical family that includes many important crops such as tomato and potato, an attractive subject for comparative genomics. Low-resolution comparative mapping indicated that *R* loci may be positionally conserved within Solanaceae (Grube *et al.*, 2000; Leister *et al.*, 1996; Pan *et al.*, 2000). To date, this knowledge has never led to the isolation of a new *R* gene with known function.

The distal end of the long arm of chromosome 11 of tomato is a particularly interesting genomic region to investigate the evolution of *R* loci in a comparative genomics perspective. First, the overall structural colinearity is excellent between tomato and potato (Tanksley *et al.*, 1992) and relatively good between tomato/potato and pepper (Livingstone *et al.*, 1999). Second, this genomic region is a hotspot for *R* genes, harboring major genes encoding resistance to the fungi *Fusarium oxysporum* (*I2*, Ori *et al.*, 1997; Simons *et al.*, 1998) and *Stemphylium spp.* (*Sm*, Behare *et al.*, 1991), and to yellow leaf curl virus (Hanson *et al.*, 2000) in tomato, to the oomycete *Phytophthora infestans* (*R3*, *R6*, and *R7*, El Kharbotly *et al.*, 1994; 1996) in potato, and to tobacco mosaic virus (*L*, Lefebvre *et al.*, 1995) in pepper. The region also contains several quantitative trait loci (QTL) conferring resistance to the cyst nematode *Globodera rostochiensis* (*Gro1.3*, Kreike *et al.*, 1993), to *P. infestans* (*phyt7*, Oberhagemann *et al.*, 1999) in potato, and to cucumber mosaic virus (*cmv4*, Grube *et al.*, 2000)and to *P. capsici* (*phyt 3*, Lefebvre and Palloix, 1996) in pepper. Last, the molecularly well characterized *I2* complex locus in tomato (Ori *et al.*, 1997; Simons *et al.*, 1998) provides an excellent template for a comparative study. The complex locus consists of two clusters, *SL8D* and *SL8E*. The *SL8D* cluster contains seven coiled-coil nucleotide binding site and leucine-rich repeat (CC-NBS-LRR) type *R* gene sequences, including the *I2* gene conferring complete resistance to race 2 of *F. oxysporum* f sp *lycopersici* (Simons et al., 1998) and the *I2C-1* (Ori et al., 1997) and *I2C-5* (Sela-Buurlage et al., 2001) genes conferring partial resistance to the same pathogen.

Potato is the most important non-cereal crop plant. One of the major

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constraints to potato production in the world is the late blight disease caused by the oomycete *P. infestans* (Fry and Goodwin, 1997). There is considerable interest in identifying late blight *R* genes and in understanding their evolution and mode of action (Ballvora *et al.*, 2002; Song *et al.*, 2003; van der Vossen *et al.*, 2003). In a previous study (Huang *et al.*, 2004), we discovered that the *R3* complex locus at chromosome 11 of potato comprises two functionally distinct late blight *R* genes, *R3a* and *R3b*. Here, we use genomic information from the model Solanaceous plant tomato to clone *R3a*, a gene that confers race-specific resistance to *P. infestans*.

Results

The potato *R3* and tomato *I2* regions are highly colinear

We previously showed that the genomic regions harboring the *R3* late blight resistance locus in potato and the *I2 Fusarium* wilt resistance locus in tomato are colinear (Huang *et al.*, 2004). To determine the extent of colinearity between these regions, we mapped seven tomato markers on the high-resolution genetic map of the *R3* complex locus (Fig. 1). All seven markers retained their order in tomato and potato. We identified a cluster of *I2* gene analogues (*I2*GAs) in potato that was mapped 0.1 cM centromeric to the TG105 marker. This potato *I2*GA cluster positionally corresponds to the *SL8D* cluster of the *I2* complex locus in tomato and was therefore named the *St-I2* cluster. The perfect micro-colinearity within the TG105-cLET24J2A interval indicates that the *R3a* cluster in potato is syntenic to the *SL8E* cluster in tomato. Despite the fact that the counterpart of the *R3b* cluster was not detected in tomato, the orthologous relationships of *SL8D* versus *St-I2* and *SL8E* versus *R3a* point to an ancient *R* locus prior to the tomato-potato divergence.

R3a candidates were identified using a local resistance gene analog (RGA) approach

Although the potato *R3a*-tomato *SL8E* synteny was established (Fig. 1), we could not directly use it for *R3a* cloning since there was no sequence available from the *SL8E* cluster. However, the *SL8E* cluster was defined by cross-hybridization with the Using this information, a local RGA approach was applied. Instead of targeting conserved motifs within the NBS applied by global RGA approaches (Aarts *et al.*, 1998; Kanazin *et al.*, 1996; Leister *et al.*, 1996; van der Linden *et al.*, 2004; Yu *et al.*, 1996), the conserved sequences within the LRR of the *SL8D* cluster were used to design the P_{SL8D} primers (Table 1C). A BLAST search (Altschul et al., 1990)for short, nearly exact matches proved the specificity of the P_{SL8D} primers, which hit no sequence other than the *SL8D* cluster with a reasonable low E-value. On DNA from the parental clones SH83-92-488 (SH) and RH89-039-16 (RH), the primers

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amplified a major smearing band of ~1kb (data not shown), indicating that many copies of *I2*GAs with similar sizes were amplified. The size fitted the prediction from the sequences of the *SL8D* cluster (884~1049bp). The above findings confirmed the suitability of the P_{SL8D} primers for identifying *I2*GAs in potato.

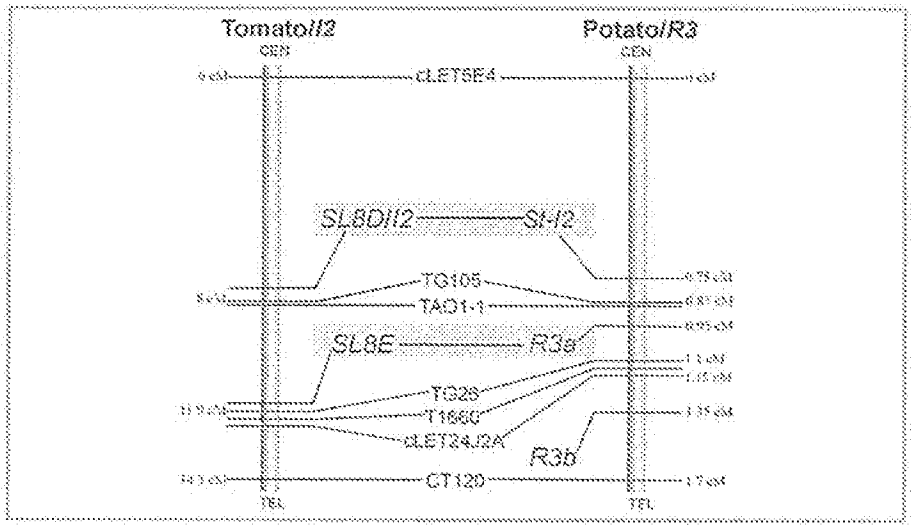


Fig. 1. Comparative genetic maps of the *I2* complex locus in tomato and the *R3* complex locus in potato. The left and right solid bars represent the tomato and potato chromosomes, respectively. Orientation is indicated by CEN (centromere) and TEL (telomere). Mapped markers are connected by black lines. The syntenic relationships of *R* gene clusters are highlighted using grey rectangles. On the tomato map, the positions of TG105, TG26, and CT120 are according to Tanksley et al. (1992), cLET5E4, T1660, and cLET24J2A according to the Solanaceae Genomics Network (http://www.sgn.cornell.edu), and *SL8D* (containing the *I2* gene), TAO1-1, and *SL8E* according to Ori et al. (1997). On the potato map, the positions of cLET5E4, TG105, *R3a*, TG26, *R3b*, and CT120 were determined by the previous work (Huang *et al.*, 2004), TAO1-1, T1660, and cLET24J2A by aligning the marker sequences to the sequences of BACs mapped on the high-resolution map of *R3* (data not shown). The position of *Sl-I2* was determined as described in text.

To identify *I2*GAs physically close to *R3a*, an association analysis on bacterial artificial chromosome (BAC) pools was conducted. Similar to other physical mapping methods like Radiation Hybrid mapping (Cox *et al.*, 1990) or HAPPY mapping (Dear and Cook, 1993), our method is based on the frequency that a given marker and *I2*GAs co-appear in BAC pools to estimate physical distance between them. The mapping panel is represented by BAC pools that contain 384 BACs, equivalent to 0.05 genome.

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Table 1. Molecular markers and primers used, aim in the study (A-flanking markers, B-physical mapping, C-RGA marker, D-RACE, and E-RT-PCR), their sequence, annealing temperature (Tm), and restriction enzyme used to generate polymorphism.

Aim	Marker	Primer (5'-3')	Tm (°C)	Restrict. enzyme
A	cLET5E4	CCA GGC ATG CTC AAT TTG GAG T TTC CCT GTT TGG ACT ACT TGT GGA	55	*Hha*I
	CT120	CGA GGG GGC GAA GGA TT CCA TGA GAT AAA CGA GGA ACC AGT	52	*Tsp*509I
B	241S	GGC ACT GAT AAG TTT TGG TTT TG GTG GTT TAT GAA TTG AAC TCA TGC	55	a.s.[c]
	194T	AAG CTT GAA TTC GTG GAC GG CTT TAT ACC AAC AGG TTG CTC	55	a.s.
	4T	AAG CTT TCA AAC CAA AAT GC AAA ATG ACT TTA CGT GGT CT	52	a.s.
	GP250	ACC AGT AGG ACC ACC ACC AAC AAT GAT CGT GAC GGC TCT ACT CTT TTA TGA	60-52[b]	*Vsp*I
	GP250-2	CTG GTA ATA GTA GTA ATG ATT CTT CGT C GAT CCT GAC TGC TCT ACT CTC TTA CGA	60-52[b]	*Vsp*I
C	$P_{6.80}$	AAT TGG AGA GTT CCC TAC ACT TGA G AGG GAG GAG GGC AGT GCT GAT TC	50	*Alu*I
D	3RACE4	CGA AAG GAG TGG CAT TTA CAG AGA CGA	68-60[b]	
	3RACE5	CTT CCT CTA TTC AAA GGC TTA CCA TAG TG	68-60	
	3RACE6	CTC ACC TCT CTT CAA TAT CTA TTT ATT AGG	68-60	
	5RACE0	CAT TGT AAA CCG CTT TAG CAA GTG TTG TC	68-60	
	5RACE4	CAT TCC AAC AAT AGG AAC TAC AGT CCG C	68-60	
	5RACE5	CAT TCT GCC TTC CAA AGA TAT CAG AG	68-60	
	5RACE6	CAT CAC TTA GCA CAA TCT GAA GAC CG	68-60	
E	RT-SH23-1	GCT TCC GAT ATG TAT TGA TCT CAC G TGT GGC AAT CCT CTA CAA ACA ATG T	68-55[b]	
	RT-R3a	ATC GTT GTC ATG CTA TGA GAT TGT T CTT CAA GGT AGT GGG CAG TAT GCT T	68-55	
	RT-SH23-3	CGA CAT GTA ATT ATT TCA TGC CTC C AGA GGA ATT TCA AAC AAG GGA GTT C	68-55	
	RT-SH194-2	TGG ATT GAA AAG TTG CCT GAT TCT G CAA GGT AGT GGG CAG TAT GCT AAA T	68-55	
	Actin[a]	CAG CAA CTG GGA TGA TAT GG ATT TCG CTT TCA GCA GTG GT	58	

[a] primers kindly provided by Chengwei Li of the Lab of Plant Breeding, Wageningen University;
[b] touchdown PCR, -1 °C/cycle;
[c] allele specific.

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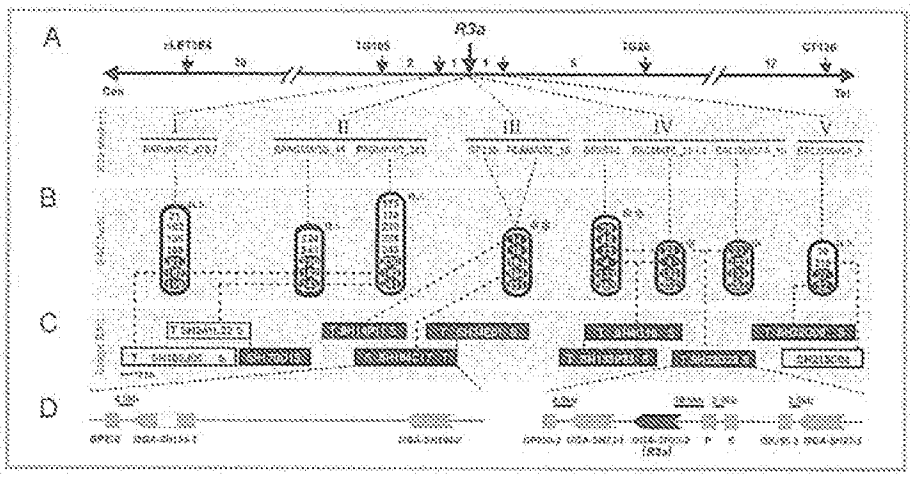


Fig. 2. Genetic and physical mapping of *R3a*.

A. Genetic map of *R3a*. Vertical arrows indicate the relative positions of markers linked to resistance. Numbers between the markers above the horizontal line indicate the number of recombinants identified in the 1,748 SHxRH progeny used in previous study (Huang *et al.*, 2004) and the additional 1,200 progeny used in this study. The *R3a* markers are divided into five groups (I-V) (described in text). GP250-2 was found in BAC pool screening, which is a copy of GP250 and also cosegregates with *R3a*.

B. Physical association of markers and *I2*GAs. Oval beads within round-end rectangles represent set of BAC pools identified by a given marker in Group I-V. BAC pools with *I2*GA(s) are represented by a black background. The extent of association (n.s.-not significant, one asterisk-$P<0.05$, two asterisks-$P<0.01$) between a given *R3a* marker and *I2*GAs on BAC pools are indicated on the upright side of each round-end rectangle.

C. BAC Contigs at the *R3a* region. Rectangles present BACs and those carrying *I2*GA(s) are highlighted using black background. T (T7) and S (Sp6) indicate the orientation of the BACs. BACs SH182J06 and SH241L22 were identified by Group I/II markers, SH14P11 and SH194C17 by Group III markers, SH4G16 and SH23G23 by Group IV markers, and SH32O08 and SH215C04 by Group V marker. SH170O15, SH24E01, and SH193P23 were identified using BAC end markers 241S, 194T and 4T (Table 1B), respectively.

D. Location and orientation of I2GAs in SH194C17 and SH23G23 are shown. Single continuous ORFs were identified in *I2GA-SH194-2, -SH23-1, -2, -3*. I2GA-SH194-1 is inserted by a retroelement (indicated by the white block). The positions of the markers GP250 and GP250-2 were identified by alignment. GP250-3 was found as another copy of GP250. The positions of the Group IV AFLP markers EACGMCAA_10 (E), and PACMATC_264.1 (P) were defined *in silico* by identifying their restriction sites, selective nucleotides, and mobilities. The distance between markers and I2GAs are indicated above the annotated BACs.

To determine whether any *R3a* marker (Fig. 2A) was physically close to an *I2*GA, we screened 255 BAC pools with the nine *R3a* markers and the P_{SLSD} primers. A total of 30 BAC pools were determined to be positive for one or more of the *R3a* markers, placing these markers in five groups on the basis of recombination events and co-occurrence in BAC pools (Fig. 2A and 2B). The P_{SLSD} primers identified 92

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*I2*GA-containing BAC pools, 19 of which coincided with those positive for the *R3a* markers. *Chi-square* tests showed that *I2*GAs were significantly associated with the markers in Group III and IV that cosegregated with *R3a* (Fig. 2B). This suggested a close physical relationship between *R3a* and specific *I2*GAs, which therefore were regarded as *R3a* candidates.

From the BAC pools identified by the *R3a* markers and by chromosome walking, we isolated 11 BAC clones that span at least 700 kb (Fig. 2C). Subsequently, BAC clone SH194C17 and SH23G23 were chosen for sequencing, as they were respectively identified by Group III and IV markers (Fig. 2C) and Southern hybridization (data not shown) indicated they contained five *I2*GAs. Annotation of the BAC sequences indeed predicted five *I2*GAs that were designated *I2GA-SH194-1*, *-2*, *I2GA-SH23-1*, *-2*, and *-3* (Fig. 2D). Single continuous open reading frames (ORF) were present in *I2GA-SH194-2* and the three *I2*GAs in BAC SH23G23. *I2GA-SH194-1* is a pseudogene due to insertion of a Ty1/Copia-type retroelement. In the BAC sequences, the GP250 marker and the amplified fragment length polymorphism (AFLP) markers could also be recognized. The physical proximity between the *I2*GAs and the Group III/IV markers (Fig. 2D) validated the predicted association at the level of BAC pools (Fig. 2B). The four *I2*GAs with full ORFs were considered as *R3a* candidates.

Table 2. Disease test of primary transformants of *R3a* and its paralogues. Numbers of plant showing resistance to the three *P. infestans* isolate IPO-0, H30P04, and 89148-09 are indicated.

Construct	Clone	# plants tested [a]	# plant showing resistance to *P. infestans* isolate [b]		
			89148-09	IPO-0	H30P04
*I2*GA-SH23-1	1029-31	12	0	0	0
	Desiree	6	0	0	0
*I2*GA-SH23-2 (*R3a*)	1029-31	8	8	8	0
	Desiree	7	7	7	0
*I2*GA-SH23-3	1029-31	13	0	0	0
	Desiree	5	0	0	0
*I2*GA-SH194-2	1029-31	23	0	0	0
	Desiree	8	0	0	0
Control	1029-31	10	0	0	0
	Desiree	10	0	0	0
	RH	10	0	0	0
	SWS540-025	10	10	10	0
	SH	10	10	10	10

[a] For constructs-number of independent transformants; for control-number of *in vitro* plantlets of the same genotype inoculated with each isolate;

[b] 89148-09 and H30P04 kindly provided by Dr. F. Govers of Lab of Phytopathology, Wageningen University and IPO-0 by Dr. W. Flier of Plant Research International, Wageningen, the Netherlands.

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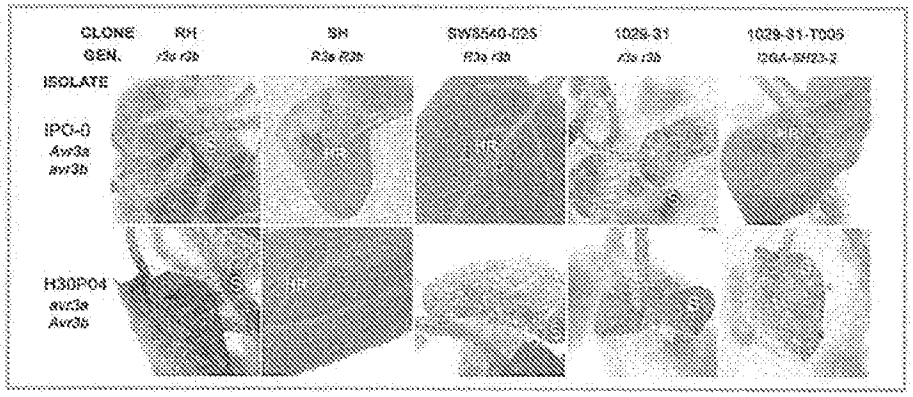


Fig. 3. *In vitro* inoculation of the primary transformants of *R3a*. Massive sporulation (S) and localized hypersensitive reactions (HR) are observed on compatible and incompatible interactions, respectively. RH and 1029-31 are susceptible to both isolates. SH is resistant to both isolates. SW8340-025 (an SHxRH progeny carrying only *R3a*) and 1029-31-T005 (a 1029-31 transformant with *I2GA-SH23-2*) are resistant to IPO-0 (with *Avr3a*) and susceptible to H30P04 (without *Avr3a*).

R3a is an *I2*GA

The genes *I2GA-SH194-1* and *-SH23-1*, *-2*, *-3* together with 2-3 kb up- and down-stream sequences were introduced into the susceptible clone 1029-31 and cv. Desiree via *Agrobacterium*-mediated transformation. At least ten *in vitro* plantlets of each primary transformant were tested for resistance to each of the three *P. infestans* isolates 89148-9, IPO-0, and H30P04 in duplicate experiments. Cv. Desiree, 1029-31, RH, SH, and SW8540-025 (*R3a* recombinant) were included as controls. Only a 10.4-kb subclone containing *I2GA-SH23-2* was able to specifically complement the susceptible phenotype. All 15 primary transformants of this subclone exhibited *R3a*-specific resistance, i.e. were resistant to isolate 89148-9 and IPO-0 and susceptible to H30P04 (Table 2 and Fig. 3). The results were confirmed using a detached-leaf assay in duplicate tests. Since the subclone contained only one ORF, we designated *I2GA-SH23-2* as *R3a*.

The transcript structure of the *R3a* gene was determined by comparing the genomic sequence with cDNA fragments generated by random amplification of cDNA ends (RACE). The *R3a* transcript is 4176 nt long and encodes a predicted polypeptide of 1,282 amino acids (a.a.) with a relative molecular mass of 145.9 kDa. The *R3a* gene and the *I2* gene (Simons *et al.*, 1998) have a similar intron-exon pattern and both have no intron in the coding region, but the intron close to the stop codon is much longer in *R3a* than in *I2* (Fig. 4A).

The *R3a* gene encodes a putative CC-NBS-LRR protein and shares 88% DNA identity and 83% amino acid (a.a.) similarity to *I2* (Fig. 4B). Considerable

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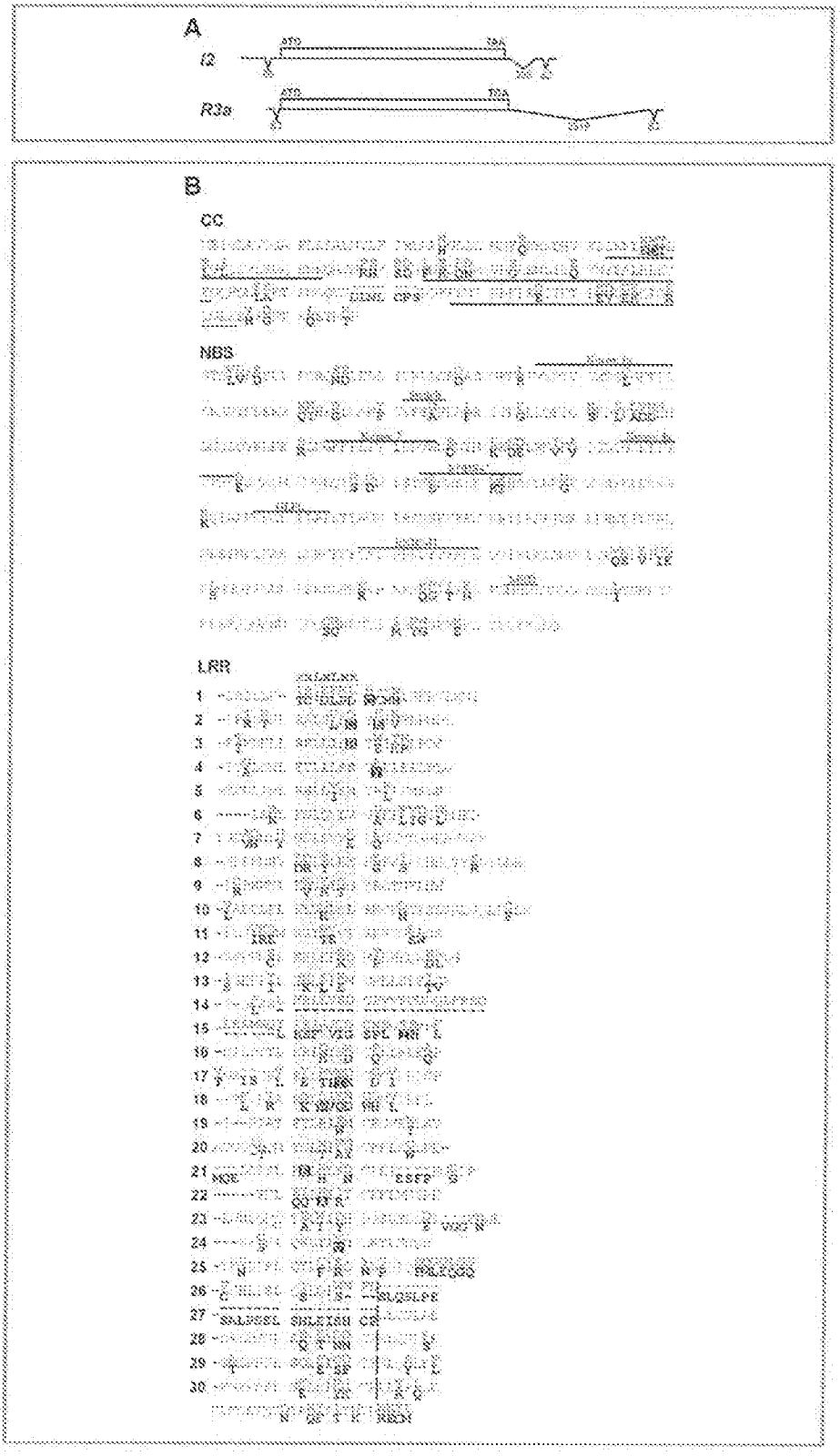
dissimilarity occurs at the CC domain, including a seven-amino acid indel. R3a and I2 proteins are quite conserved in the NBS domain (86% a.a. identity), especially at the motifs that define the domain (94% a.a. identity). The proteins mainly diverge at the LRR domain where two major differences were observed. First, although both proteins carry 29 LRR units, the R3a protein lacks a complete LRR unit of 28-amino acids at LRR 14/15 and contains an extra copy of a unique LRR unit of 23-amino acids at the LRR 26/27 (Fig. 4B). Second, 45% (63/140) of the putative solvent-exposed residues (x in the frame xxLxLxx) differ between the I2 and R3a proteins.

The R3a and I2 proteins are more related to each other than to other known R proteins, as indicated by the zero E-value using BLASTP (Altschul *et al.*, 1990). Thus the *I2* and *R3a* genes belong to the same *R* gene family. Outside of this family, the most related known *R* gene is *Rpg1-b* ($E=e^{-176}$, 35% a.a. identity) from soybean mediating recognition of the Type III effector protein AvrB from *Pseudomonas syringae* (Ashfield *et al.*, 2004). The R3a protein bears only limited similarity (15% and 30% a.a. identity) to the other two known late blight R proteins, R1 (Ballvora *et al.*, 2002) and RB/Rpi-blb1 (Song *et al.*, 2003; van der Vossen *et al.*, 2003), respectively.

Dynamic evolution occurred in the *R3a* cluster

Despite the excellent overall colinearity in the *I2* and *R3* genomic regions (Fig. 1), the *R3* region is physically larger in potato. In tomato the TG105-TG26 interval spans about 500 kb and contains one or few *I2*GAs, as demonstrated by long-range physical mapping (Simons et al., 1998) and Southern analysis (Ori *et al.*, 1997). In contrast, the TG105-TG26 interval in SH spans more than 1 MB and contains at least nine *I2*GAs. The three contigs (11 BACs in total) in the *R3a* region (Fig. 2C) span at least 700 kb. In addition, gaps between the depicted contigs and those between the depicted contigs and contigs harboring the markers TG105 and TG26 are predicted to be larger than 100 kb, the average insert size of the SH BAC library. In addition to the five *I2*GAs in BAC clone SH194C17 and SH23G23, we identified a new *I2*GA in each of the four BACs (SH170O15, SH24E01, SH193P23, and SH32O08, Fig. 2C) by Southern blotting (data not shown). Altogether, these data indicate that the *R3* complex locus in SH has expanded significantly compared to that of tomato (cv. Motelle and Mogeor, Ori et al., 1997; breeding line E22, Simons et al., 1998), resulting in an increase in the number of *R* gene sequences. It remains unknown whether such variation in size and *R* gene copy number reflects *de facto* tomato and potato genomes or whether such variation also exists within each species.

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⟸ **Fig. 4.** Comparison between *R3a* and *I2*.

A. Schematic diagram of the transcript structures of *I2* and *R3a*. Horizontal lines indicate exons. Open rectangles represent coding sequences. Lines angled downwards indicate the position of introns, whose sizes are indicated below.

B. Primary structure and alignment of the I2 and R3a resistance proteins. Amino acid residues of I2 and R3a that are identical are shown in normal script. I2- and R3a- specific residues are shown in bold at top and bottom lines, respectively. Dashes indicate deletion. The predicted coiled coils (Lupas *et al.*, 1991) in the CC domains are underlined. The conserved motifs of the NBS domain are overlined. The 30 LRR units in the alignment are numbered. The β-sheet (consensus xxLxLxx) of each LRR is highlighted with a grey background. The R3a residues under significant diversifying selection are highlighted with black background. The calculation is based on the alignment of *R3a*, *I2GA-SH23-1*, *-3*, and *I2GA-SH194-2* using PAML (Yang *et al.*, 2000). Between vertical lines are the unique LRR units (consensus NLQSLAESALPSSLSHLEIDDCP) that display distinct copy numbers between *I2*, *R3a*, and their paralogues.

Diversifying selection has been detected in many *R* gene complex loci (Hulbert *et al.*, 2001). Sites under diversifying selection were investigated using the program PAML (Yang et al. 1997; 2000). Models M7 and M8 in the program "codeml" of PAML were run for the four *I2*GAs (*R3a*, *I2GA-SH23-1*, *-3*, and *I2GA-SH194-2*) at the *R3a* cluster in potato. Model M7 is a special case of Model M8 and assumes no selection, whereas Model M8 allows for positively selected sites (Yang et al., 2000). Diversifying selection can be confirmed using a likelihood-ratio test by comparing the likelihood calculated using models M8 and M7 (Yang et al., 2000). Comparison of the results from Model 7 and Model 8 showed that the I2GAs at the *R3a* cluster have undergone diversifying selection ($\chi^2 = 92.8$, df = 2; $p<0.001$). Selection at each site of the *R3a* homologues was calculated using model M8. Thirteen sites were found to be under significant diversifying selection (Fig. 4B and Fig. 5). Twelve of them are in the LRR domain and seven are putative solvent-exposed residues. This observation is compatible with the idea that the LRR region of an R protein mainly defines recognition specificities (Parniske *et al.*, 1997; Van der Hoorn *et al.*, 2001; Wulff *et al.*, 2001).

Putative sequence exchange between paralogues was previously observed at several *R* gene complex loci (Noel *et al.*, 1999; Parniske *et al.*, 1997) and is likely a mechanism for creating new specificities (reviewed in Hulbert *et al.*, 2001). Sequence relationships within the *R3a* cluster were analyzed by determining informative polymorphic sites (IPS, Parniske *et al.*, 1997). In total, 129 IPS were detected (Fig. 6). The *R3a* and *I2GA-SH23-1* genes have 94% DNA identity and they share 91 IPS, suggesting that they are derived from the same ancestral gene. Interestingly, in the central part of the alignment, the *R3a* cluster exhibits a complex patchwork. The *R3a* and *I2GA-SH194-2* genes share sequence affiliations at three patches, comprising 21 IPS, which result in 10 non-synonymous substitutions

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between *R3a* and *I2GA-SH23-1*. An almost continuous sequence affiliation (nine IPS) between the *R3a* and *I2GA-SH23-2* genes was also found, comprising three non-synonymous substitutions. It remains unclear whether the putative sequence exchanges between the paralogues have led to new resistance specificities in the *R3a* cluster.

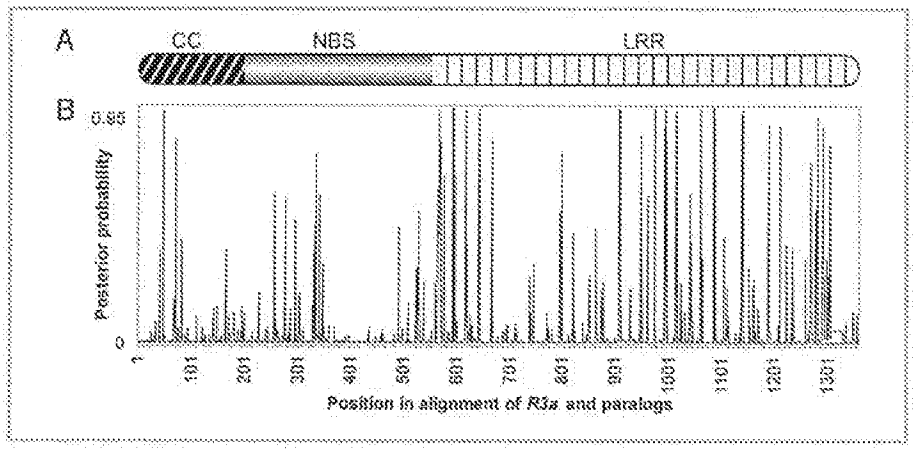


Fig. 5. The distribution of codons under diversifying selection in *R3a*.

A. The horizontal bar schematically represents the R3a protein. Sizes of the three domains are indicated by the codon position on the *x* axis below.

B. The posterior probability (PP) of diversifying selection at each site in R3a and paralogues is plotted. A site with PP>0.95 is considered to be under significant diversifying selection.

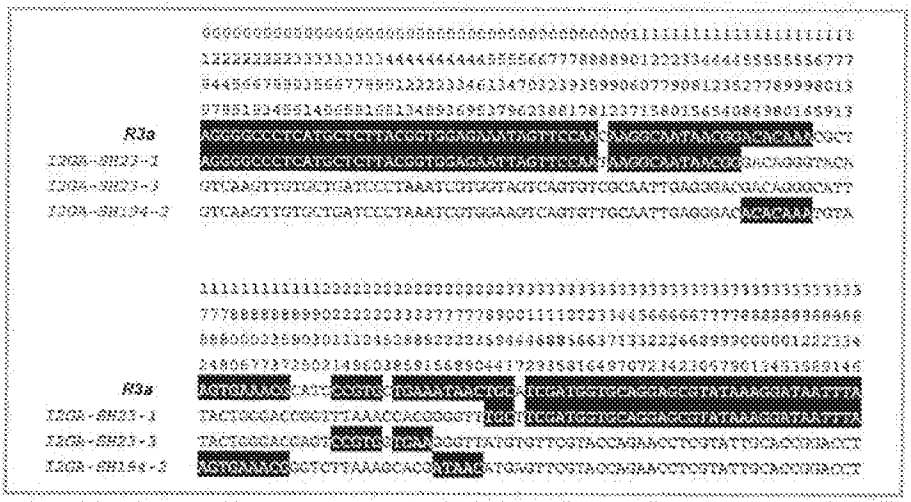


Fig. 6. Sequence relationships between members of the *R3a* cluster. Display of all 129 IPS (a polymorphic nucleotide which is shared between two *I2GAs* within the alignment of four). The position of each site relative to the first nucleotide of the ATG start codon within the alignment is given by vertical numbers above each site. Highlighted are sequence patch of at least three consecutive IPS that shared between *R3a* and one of the other three *I2GAs*.

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R3a is constitutively expressed

To examine the expression patterns of the genes *R3a*, *I2GA-SH23-1*, *-3*, and *I2GA-SH194-2*, gene-specific semi-quantitative RT-PCR was performed on mRNA isolated with from *P. infestans* challenged (isolate IPO-0 and 90128) or mock inoculated leaves of SH at 0, 8, 16, 24, 32, 48, and 72 hours post inoculation. All the four *I2*GAs are constitutively expressed in all treatments, but the expression level of *I2GA-SH23-3* is significantly lower than the other paralogues (Fig. 7).

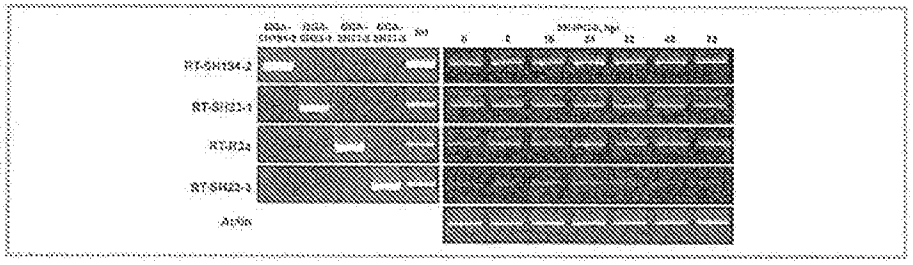


Fig. 7. RT-PCR of the *R3a* cluster. Gene-specific primers used are listed on the left. Subclones with individual *I2*GAs (*I2GA-SH194-2*, *-SH23-1*, *-2*, *-3*) and genomic DNA of SH are used as control for the specificities of the primers. mRNA isolated from the detached leaves of SH inoculated with *P. infestans* isolate IPO-0 were used to study the expression pattern in a time course from 0 to 72 hpi. Actin primers were used to monitor mRNA quantity.

Discussion

In this study, we used genomic information from tomato to isolate the potato late blight resistance gene *R3a* from an ancient locus involved in plant innate immunity in the Solanaceae. Despite technological advances in genomics in recent years, positional cloning of *R* genes from large-genome crop species is still far from a routine procedure mainly due to low recombination frequencies and the high repetitive nature that are characteristics of most complex *R* loci (Hulbert *et al.*, 2001). In potato, the heterozygous genome is an additional complicating factor (Kanyuka *et al.*, 1999). To partially circumvent these obstacles, our local RGA approach analyzes genetic markers on BAC pools or superpools, offering enough template complexity in PCR to allow most markers to be allelic- and locus-specific as compared to the hybridization method on single BAC filters. Although the RGA specific primers (P_{SLRD}) were designed for the exclusive identification of the *I2* gene family, they were also designed to be allele- and locus-insensitive in order to include all candidate RGAs at the target region. Through association on BAC pools, we could identify a subset of RGAs that were in coupling phase with and physically close to genetic markers linked to *R3a* and thus 'jump' rather than 'walk' into BACs carrying such RGAs. The success of the method depends on the marker saturation level at the

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target region and the capacity of the RGA specific primers to amplify all the candidate RGAs at the target region. In this case, we benefited from the marker saturation provided by the ultra-high dense (UHD) AFLP potato map (Isidore *et al.*, 2003), since the same mapping population (SHxRH) was used to generate the UHD map and for the initial mapping of *R3a* (Huang *et al.*, 2004). In general, a bulked segregant analysis (Michelmore *et al.*, 1991) in combination with AFLP marker technology will generate adequate marker saturation of the *R* gene regions since the 'wild' *R* haplotype often differs a lot from the 'domestic' *r* haplotype, which is also the reason that they hardly recombine. The synteny at the target region between tomato and potato was beneficial to the RGA approach, despite extensive expansion of the *R3* complex locus compared to the syntenic *I2* complex locus.

Most of the molecularly characterized *R* genes belong to families of tightly linked genes and at many such complex loci, most paralogues appear to encode proteins similar to the functional *R* genes (Hulbert *et al.*, 2001). We demonstrated that all four paralogues of the *R3a* cluster are constitutively expressed (Fig. 7), even in unchallenged plants. It is unclear whether these paralogues are capable of interacting with unknown elicitors or whether they are just relics of a recent 'birth-and-death' process (Michelmore and Meyers, 1998). Given the fitness cost of *R* gene expression (Tian *et al.*, 2003), unnecessary *R* genes should undergo a 'death' process. We also found many paralogues that are truncated, mutated via frame-shift (data not shown), or inserted by a retroelement (Fig. 2D). The weaker expression of *I2GA-SH23-3* (Fig. 7) might suggest that mutations accumulated in the promoter region can lead to a lower expression and thus eventually to the 'death' of the gene. Alternatively, the expressed paralogous proteins may play a role through heteroduplex formation with R3a, as inter- and intramolecular interactions between R protein domains may function as activation switches upon recognition of Avr elicitors (Moffett *et al.*, 2002).

Plants can not move to escape their microbial environment. To combat disease, plant have developed a sophisticated innate immunity system, where *R* genes play a central role (Dangl and Jones, 2001). Comparative genomics may provide insight into how diseases have resulted in differential evolution of *R* loci between closely related plant species and here we show an example. The potato *R3* region has undergone a significant physical expansion compared to the syntenic tomato *I2* region. This expansion may have resulted in functional diversification. For instance, at least 10 *I2*GAs have been found at the *R3b* region (S. H., H. K., et al., unpublished work), suggesting *R3b* could also be an *I2*GA. The contrasting evolutionary fates of the ancient *I2-R3* complex locus in the closely related tomato and potato genomes are consistent with the opposite evolutionary potentials of the

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interacting pathogens (McDonald and Linde, 2002). *Fusarium oxysporum* f. sp. *lycopersici* is a soilborne fungus with low genotype diversity, whereas the late blight pathogen *P. infestans* is notorious for its ability to move and mutate (McDonald and Linde, 2002). The great evolutionary potential of *P. infestans* may have stimulated the interacting *R3* complex locus to expand its *R* gene repertoire, as supported by the observed physical expansion and the fact that two functionally distinct genes locate in the locus (Huang *et al.*, 2004). *P. infestans* also infects tomato, but genomic regions controlling late blight resistance show very limited overlap between tomato and potato (Brouwer *et al.*, 2004; Gebhardt and Valkonen, 2001; Grube *et al.*, 2000). Late blight resistance in the two closely-related species is likely conferred by different loci that have evolved independently.

Plant disease resistance genes display two distinct evolutionary patterns contrasting at the rate of evolution (Kuang *et al.*, 2004). The late blight *R* gene *RB/Rpi-blb1* from *S. bulbocastanum* belongs to the Type II (slow evolving) *R* gene class (Song *et al.*, 2003). However, the occurrence of sequence exchanges between paralogues (Fig. 6), the multi-allelic nature of the locus (see below), high nucleotide identities between homologues (90-94%) and obscure allelic/orthologous relationships between the SH *R3* haplotype and other *S. demissum* haplotypes (H.K., S.H., et al., unpublished work) lead to the putative classification of *R3a* into the Type I (fast evolving) class. It remains unknown why these two late blight *R* genes differ by the rate of evolution, but there are some hints. *RB/Rpi-blb1* is resistant to all tested races of *P. infestans* and provides resistance by reducing the infection rate (Song *et al.*, 2003; van der Vossen *et al.*, 2003), while *R3a* is a race-specific gene and displays a typical hypersensitive necrosis response (Huang *et al.*, 2004). *S. bulbocastanum* often grows under quite dry conditions (Hawkes, 1990), suggesting the absence of co-evolution with *P. infestans*, whereas *S. demissum* has co-evolved with the pathogen on the cool mountain-forests in Toluca Valley of Mexico (Rivera-Pena, 1990), which was recognized as a center of diversity for *P. infestans*.

An intriguing question is why *S. demissum* appears to contain only easily-broken *R* genes (Wastie, 1991) but displays durable resistance at the population level (Rivera-Pena, 1990). The polymorphism of parasite recognition capacity in a host population will restrict most isolates of the parasite to grow on most hosts (Hamilton *et al.*, 1990). Allelism is an efficient way of creating recognition polymorphism (Bergelson *et al.*, 2001), and we are currently investigating the multiple allelism of the *R3* complex locus. (S. H. et al., in preparation), which might be a natural mechanism of *S. demissum* to suppress late blight epidemics, similar to the concept of *R*-gene polycultures or multilines (Jones, 2001; Mundt, 2002). The isolation of *R3a* and characterization of the *R3* complex

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locus will facilitate the cloning of other alleles and thus provide a platform to test the *R*-gene polyculture concept in late blight disease management in commercial potato production.

Materials and Methods

Plant material

The F1 population of SH83-92-488 (SH) x RH89-039-16 (RH) that segregates for both *R3a* and *R3b* was used for genetic mapping (Huang *et al.*, 2004). SW8540-025 is a recombinant that only carries *R3a*. The susceptible potato clones 1029-31 and cv. Desiree were used for genetic transformation.

P. infestans isolates and inoculation

P. infestans isolates 89148-09 (*Avr3a*, *Avr3b*), IPO-0 (*Avr3a*, *avr3b*), H30P04 (*avr3a*, *Avr3b*), and 90128 (*avr3a*, *avr3b*) were inoculated on *in vitro* plantlets (S. H. et al., submitted) or detached leaves (Huang *et al.*, 2004).

Genetic mapping

A total of 3,000 SHxRH progeny were screened for recombinations in the *R3a* region using the flanking markers cLET5E4 and CT120 (Table 1A). The recombinants were used to determine the position of the *R3a* markers (Fig. 1 and Fig.2A). DNA isolation and analysis of molecular markers were described previously (Huang *et al.*, 2004).

Physical mapping

A 10-genome equivalent bacterial artificial chromosome (BAC) library of the resistant parent SH, stored in 255 384-well microtitre plates, was screened with the *R3a* markers stepwise: first, positive superpools (1 superpool=8 BAC pools) were identified; second, positive pools were identified from those positive superpools. Plasmid DNA was isolated using the standard alkaline-lysis protocol from pooled bacteria to produce 255 BAC pools. Screening of the BAC pools and identification of single positive BACs were performed as described previously (Rouppe van der Voort *et al.*, 1999).

Plasmids of single BACs were purified using MIDI-PREP columns (Qiagen, Hamburg, Germany). Sequences generated through BAC-end sequencing with SP6 or T7 primers were employed to design PCR primers to develop BAC end markers. The BAC end markers were named according to the BAC pool number followed by S (SP6) or T (T7) (Table 1B). In view of the repetitive nature of the *R3* complex locus, the overlap of two BACs was verified by: 1) *Hin*dIII restriction mapping, 2) non-selective AFLP fingerprinting using *Hin*dIII and *Mse*I/*Taq*I (Simons *et al.*, 1998), and 3) analysis of BAC end markers through Tm-gradient PCR, restriction with 24 frequent cutters, and sequencing of the PCR products. The length of overlapping between two BACs was determined by adding the sizes of co-migrating *Hin*dIII restriction fragments.

DNA sequencing and analysis

The DNA sequences of the BAC clones SH194C17 and SH23G23 (Fig. 2D) were determined by using a shotgun sequencing strategy (van der Vossen *et al.*, 2000). Positions of putative genes were predicted using GENSCAN (Burge and Karlin, 1997) and GENEMARK (Lukashin and Borodovsky, 1998). Multiple sequence alignments were conducted by using CLUSTALX 1.81 (Thompson *et al.*, 1997), informative polymorphic sites (IPS, Parniske *et al.*, 1997) were determined. Diversifying selection was investigated using PAML (Yang, 1997; Yang et al., 2000). Models M7 and M8 in program "codeml" of

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PAML were run for all *I2*GAs at the *R3a* cluster. Diversifying selection was confirmed using a likelihood-ratio test by comparing the likelihood calculated using models M8 and M7 (Yang et al., 2000). Selection in each site of the *R3a* homologues was calculated using model M8.

Subcloning and transformation of *I2*GAs

Candidate *I2*GAs were subcloned into the binary vector pBINPLUS (van Engelen *et al.*, 1995) as described previously (van der Vossen *et al.*, 2003). Binary plasmids harboring the candidate genes were transformed to *Agrobacterium tumefaciens* strain AGL0 (Lazo *et al.*, 1991) and introduced into 1029-31 and cv. Desiree according to standard protocols (Visser *et al.*, 1991).

Transcript analysis of the *R3a* cluster

Total RNA was extracted from detached leaves of SH inoculated with *P. infestans* IPO-0, 90128, and water using TRIZOL (Invitrogen, Carlsbad, CA, USA). The mRNA was isolated from the total RNA samples using the OLIGOTEC kit (Qiagen, Germany). The 5'- and 3'-ends of the *R3a* transcript were determined by rapid amplification of cDNA ends (RACE) using the MARATHON kit (BD Bioscience, USA) with nested gene specific primers (3RACE4-6 and 5RACE4-6, Table 1D). The cDNA for 5' RACE was synthesized with the primer 5RACE0. PCR products were cloned into pGEM-T vector (Promega, Madison, WI, USA). For gene-specific RT-PCR (Table 1E), an aliquot of 0.1 µg mRNA from each sample was used to generate single-stranded cDNA using SUPERSCRIPT II (Invitrogen, Carlsbad, CA, USA). Actin primers were used to monitor mRNA concentration (Table 1E). Genomic DNA of SH and RH and BAC subclones were used as control. Gene-specific amplification was confirmed by sequencing.

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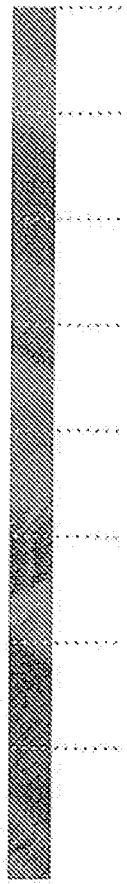
Chapter 5

High Allelic Diversity of the Potato *R3* Complex Locus Suggests A Novel Strategy for Late Blight Control

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Abstract

The oomycete pathogen *Phytophthora infestans* was the cause of the Irish potato famine in 1845 and is still a major threat of the most important non-cereal crop production in the world. Since the beginning of the 20th century, great effort was put into the introgression of single dominant resistance (*R*) genes from wild species, particularly from *Solanum demissum*. The process was ever promising but ended up into disappointment, because these race-specific *R* genes (*R1-R11*) only provided short-lived protection in the field when deployed separately. Despite the fact that single *S. demissum R* genes are not durable, it has been shown that this wild species displays a durable resistance at population level under natural conditions at the Toluca Valley of Mexico, which is considered as a center of diversity of the pathogen. Here we demonstrate that except *R1*, *R2* and *R4*, all other characterized genes (*R5-11*) are or contain allelic versions of the *R3* complex locus. The unexpected discovery reveals that the potential of resistance breeding is not fully explored and implicates that the multiple allelism of the *R3* locus could be a putative natural mechanism for late blight control in the wild populations of *S. demissum*.

Introduction

Potato breeding for late blight resistance was one of the earliest mankind practices in combating plant pathogens by means of genetic improvement (Müller and Black, 1952), but the disease has not been controlled by resistance breeding so far. The ravages of the disease observed in the Great Irish Famine in the middle of 19th century immediately stimulated the search and breeding for new resistant cultivars. However, resistance breeding in real sense didn't start until in 1909 Salaman (1929) discovered resistance (*R*) genes from the wild species *Solanum edinense* (2N=5x=60), which is a natural hybrid between *S. tuberosum* (2N=4x=48) and *S. demissum* (2N=6x=72). The fact that *S. demissum* can cross with *S. tuberosum* either directly or via *S. phureja* (2N=2x=24) made the hexaploid the major donor of *R*

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genes. Thus far 11 distinct resistance specificities (*R1-11*) (Black et al., 1953; Malcolmson and Black, 1966; Malcolmson, 1969) derived from *S. demissum* were characterized as dominant race-specific *R* genes and they interact with the late blight oomycete pathogen *Phytophthora infestans* in a gene-for-gene manner (Black et al., 1953; Flor, 1971; van der Lee et al., 2001a). Unfortunately, resistance breakdown was found in cultivars carrying one or few *R* genes in monoculture (Möller and Black, 1952) and *P. infestans* isolates that can infect all the 11 specificities were occasionally found (Wastie, 1991). Disappointed by the non-durable nature of the *S. demissum R* genes, researchers and breeders 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 so-called '*R*-gene-free' approaches to explore partial resistance (Turkensteen, 1993; Landeo et al., 1995). However, both alternatives appeared to be problematic: sexual barriers made the flow of *R* genes from other wild species time-consuming and often partial resistance is unfavorably linked to late maturity and low vigour (Collins et al., 1999; Simko, 2002). Currently, disease management of late blight heavily relies on intensive spray of pesticides, which raises considerable environmental concern and is too costly to farmers in developing countries. In Western Europe and USA where the majority of potato breeding activities occurs, late blight resistance breeding is at the atmosphere of disappointment. It is the time now to revisit the strategy adopted by breeders.

The 'boom-bust' cycles observed in the potato-*P. infestans* system is not a unique phenomenon but rather a general rule of many gene-for-gene pathosystems in monocultures (Pink and Puddephat, 1999). To avoid to be locked into such cycles, there are basically two alternatives in *R* gene deployment: *R* gene pyramiding and *R* gene polyculture. *R* gene pyramiding is actually the strategy adopted by potato breeders. This resulted in the release of cultivars with several *R* genes, for example the Dutch cv. Escort containing *R1-3* and *R10*. However, such cultivars so far do not grow in a considerable acreage, possibly because of the linkage between resistance genes and unfavorable 'wild' traits. *R* gene polyculture or cultivar mixture is another alternative solution to the vulnerability of monocultured crops to disease (Wolfe, 1985; Jones, 2001; Mundt, 2002). A large-scale mixture of a susceptible cultivar of rice with a resistant one rendered 25 fold reduction in blast incidence in the susceptible cultivar as compared to its performance in monoculture (Zhu et al., 2000). A similar study suggesting that cultivar mixtures can reduce disease incidence has been reported for late blight resistance in potato (Garrett and Mundt, 2000). However, cultivars differ not only in *R* genes but also in other agronomic characteristics such as harvest maturity or consumer preference, resulting in the reduced profitability of crops that are grown in mixtures. It is tempting to create an *R*

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gene polyculture (Jones, 2001) that is uniform in all agronomic traits but recognition specificities to *P. infestans*. In self-fertilizing seed propagated crops as tomato, this is applicable by means of conventional near-isogenic backcross lines but almost impossible in potato, a clonally propagated auto-tetraploid that seriously suffers from inbreeding depression (Ross, 1986).

The genetic modified organism (GMO) approach is so far the only possible approach to effectively practice *R* gene pyramiding and/or polyculture in potato late blight control. Potato is amenable for *Agrobacterium*-mediated genetic transformation (Visser et al., 1991) and recently marker-free GMO techniques have been developed to kick out the antibiotic or herbicide resistance gene (de Vetten et al., 2003). Using GMO, the problems such as linkage drag and inbreeding depression that are inherited to conventional potato breeding can be circumvented. The recent isolation of three late blight *R* genes, namely *R1* (Ballvora et al., 2002) and *R3a* (**Chapter 4**) derived from *S. demissum* and *Rb/Rpi-blb1* from *S. bulbocastanum* (Song et al., 2003; van der Vossen et al., 2003), provides the starting point to apply the GMO approach in either *R* gene pyramiding or polyculture strategies. However, either strategy requires more cloned *R* genes, since the *R1* and *R3a* genes have already been intensively used in cultivars and the corresponding virulent *P. infestans* strains are widely spread.

In previous work (Huang et al., 2004, **Chapter 4**), we discovered that one allelic version of the *R3* complex locus at the chromosome 11 of potato comprises two functionally distinct late blight *R* genes, *R3a* and *R3b*, and we isolated the *R3a* gene. In the present study, we report an unexpected discovery that the *R3* locus contains multiple allelic versions, including the *R5-R11* specificities. This finding, together with our previous investigations, will make it possible to isolate a dozen *R* genes in the near future for the GMO approach. Furthermore, the multi-allelic nature of the major late blight resistance locus suggests that *R* gene polyculture is likely the natural mechanism of late blight control adopted by wild populations of *S. demissum*, from which all the *R3* allelic versions were derived.

Results

The *R3* complex locus has multiple allelic versions

The late blight genes *R3*, *R6*, and *R7* co-localize on the distal end of chromosome 11 in potato (El Kharbotly et al., 1994; 1996) and are likely allelic to each other. By pedigree analysis, we identified five different classes of R3 cvs. or clones with independent pedigrees: Sc *R3* derived from CPC2127, SH *R3* from Dms 29, Ma *R3* from Dms 49, MPI *R3* from MPI19268 that also carries *R10*, and FS *R3* present in potato cvs. Forelle and Saphir that have several *S. demissum* accessions in their

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Table 1. Plant materials used in the study,the species, the known R genes and pedigrees.

RN85-035-18	*ly*	-	-	*R3* mapping male parent
SH83-92-488	*ly*	SH *R3* (Rla, *3b*)	dms 29	*R3* mapping female parent
SH83-92-018	*ly*	*R3a*	SH83-92-488	*R3a* recombinant
SH83-92-021	*ly*	*R3b*	SH83-92-488	*R3b* recombinant
Money Maker	*ly*	-	-	
Sw^4 *R0*	*ly*	-	-	Syn. to Sweet
Sw^5 *R0*	*ly*	-	-	Syn. to Craig Royal
1024-2	*ly*	-	-	
83-1009-01	*ly*	-	-	
Glacier	*ly*	-	-	
Katahdin	*ly*	-	-	
Pennycross	*ly*	-	-	
Rutgers	*ly*	-	-	
Stevens	*ly*	-	-	
Sunpeak	*ly*	-	-	
BLB356-1	*Rb*	*Rpi-blb3*	-	
BLB356-11	*Rb*	*Rpi-blb2*	-	
CGN17805	*Rb*	R_{cph}	-	
CGN21358	*Rb*	R_{vnt1}	-	
Ma *R0*	*ly*	*R1*	dms	Syn. to Caldwell 5136 S
Sc *R1*	*ly*	*R1*	CPC2127	Syn. to Craig Simonite
Ma *R2*	*ly*	*R2*	dms 54	Syn. to Caldwell 4158-4
Sc *R2*	*ly*	*R2*	CPC2127	Syn. to (912 of 16)
Ma *R4*	*ly*	*R4*	dms 29	Syn. to Caldwell 4331-5
Sc *R4*	*ly*	*R4*	CPC2127	Syn. to (565 of 34)
CPC2127	*dms*	*R1 R2, R3, R4, R5, R6*	-	
Sc *R3*	*ly*	*Sc R3*	CPC2127	Syn. to Pastorale New
Augusta	*ly*	*Sh R3*	CPC2127	
Bonita	*ly*	*Sa R3*	CPC2127	
Carnival	*ly*	*Sh R3*	CPC2127	
Starmaker	*ly*	*Sa R3*	CPC2127	
692-5466-5	*ly*	*Sh R3*	CPC2127	disrupted derivative of Carnival
Manitou	*ly*	*Sa R3, R1*	CPC2127	
Petrovsk	*ly*	*Sa R3*	CPC2127	
CGN17417-016	*ly*	*SH R3, R1.2*	dms 29	
CGN21106-005	*ly*	*SH R3, R1.3*	dms 29	
Carrick	*ly*	*SH R3*	dms 29	
Sweet	*ly*	*SH R1, R1b, R1.3*	dms 29, CPC2127	
391-6764-21	*ly*	*SH R3*	dms 29	disrupted derivative of Sweet
391-6764-68	*ly*	*SH R3*	dms 29	disrupted derivative of Sweet
Reed	*ly*	*SH R3, R1*	dms 29	
Rosita	*ly*	*SH R3, R1*	dms 29	
Stevens	*ly*	*SH R3, R1*	dms 29	
Ma R3	*ly*	*Ma R3*	dms 45	Syn. to Caldwell 5136-1
Apex	*ly*	*MPI R3*	MPI10808	
Redskin	*ly*	*MPI R3*	MPI10809	
Porcire	*ly*	*PB R3*	Several dms lines	
Pola	*ly*	*PS R3*	Several dms lines	
Saturn	*ly*	*PB R3*	Several dms lines	
397702-1-1	*ly*	*PS R3, R1*	Several dms lines	
Ma R6	*ly*	*R6, R6-1, R6-2* [a]	Unknown	Syn. to x02-21
391-6601-57	*ly*	*R6*	Ma R6	disrupted derivative of Ma R6
Ma R7	*ly*	*R7*	CPC2127	Syn. to 2192 of 7)
692-6701-108	*ly*	*R7*	Ma R7	disrupted derivative of Ma R7
Ma R8	*ly*	*R8*	CPC2127	Syn. to (804 of 8)
Ma R9	*ly*	*R9, R9.2*	CPC2127 and others dms lines	Syn. to (927 of 9)
Ma R5	*ly*	*R5*	self Ma R5	Syn. to 2005.15
Ma R10	*ly*	*R10*	MPI10755	Syn. to (661 of 10)
Amigo	*ly*	*R10*	MPI10808	
Arica	*ly*	*R10*	MPI10809	
Jaerla	*ly*	*R10*	MPI10808	
Xandia	*ly*	*R10*	MPI10809	
Flamenco	*ly*	*R10*	MPI10808	
Producent	*ly*	*R10*	MPI10809	
Promenent	*ly*	*R10*	MPI10808	
Rondo	*ly*	*R10*	MPI10809	
Sonia	*ly*	*R10*	MPI10808	
Ma R11	*ly*	*R11*	Several dms lines	Syn. to 5003 andb
4205-1	*ly*	*R11*	Ma R11	

[a] Two sets of *R* gene differentials are used internationally, namely the Dutch differential set (Ma, used by Mastenbroek) and the Scottish differential set (Sc, used by Black). These two sets differ by their *R0-R4* differentials but use the same *R5-R11* differentials.

b Species: *lyc-Solanum lycopersicum, blb-S. bulbocastanum, dms-S. demissum, tbr-S. tuberosum*

[c] Three genes (*R6, R6-1*, and *R6-2*) have been found in Ma *R6* by analysis of a segregating population from the cross Ma *R6* x Katahdin (data not shown). These three genes are independently inherited. R6 was the one that 391-6601-57 contains and was mapped at the *R3* complex locus.

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pedigree (Table 1). The resistance specificities within these cvs. and clones were assigned *R3* based on their phenotypes with a limited number of common *P. infestans* isolates (Black et al., 1953), but the *R3* genes they carry may not be identical and could be allelic versions. The above information implied that the *R3* complex locus might have more allelic versions.

To test this hypothesis, we analyzed a collection of 67 genotypes (Table 1) with 25 amplified fragment length polymorphism (AFLP) and cleaved amplified polymorphic sequence (CAPS) markers (Table 2) that cover a genetic interval of 5.5 cM at the *R3* region in the clone SH (Huang et al., 2004). Based on fingerprinting, we divided the genetic interval into two sub-intervals (SI) (Table 2). SI1 was spanned by the upper five markers that are loosely associated with each other and with the markers below. SI2 included the remaining 19 markers that define the region of the complex *R3* locus, which consists of three *R* gene clusters, namely the *St-I2* cluster that is syntenic to the tomato *Fusarium* wilt *I2* gene cluster, the *R3a* cluster, and the *R3b* cluster (Huang et al., *submitted*). We classified the 67 genotypes into three groups based on their marker pattern at SI2 (Table 2). Group I included the tomato cv. Moneymaker, four *S. bulbocastanum* accessions, 10 *S. tuberosum* cvs. and clones that have no traceable pedigree from *S. demissum*, and the Dutch (Ma, used by Mastenbroek) and Scottish (Sc, used by Black) *R1*, *R2*, and *R4* differentials; Group II consisted of all clones carrying different *R3*s, *R6*, *R7*, *R8*, and *R9* genes, and Group III comprised the *R5*, *R10*, and *R11* clones and cvs. Group I, II, and III shared 0~16%, 79-100%, and 37~68% marker alleles with the reference clone SH at SI2, respectively. The contrasting marker patterns of Group I with Group II and III confirmed that tomato (cv. Moneymaker) and old *S. tuberosum* cvs. have no *R3* allele and that *R1*, *R2*, and *R4* are independently inherited from *R3* (Müller and Black, 1952). Together with pedigree data, the fingerprinting showed that genotypes in Group II and Group III carry chromosomal fragments that were introgressed from *S. demissum* and are similar to the corresponding region in SH. In the Group III genotypes, absence of marker alleles was observed at the *R3a* region, suggesting that insertion/deletions occur or the region has similarity with *S. tuberosum* alleles. The marker pattern in Table 1 indicated *R* genes of Group II and III genotypes at SI2 are either allelic or identical to the SH *R3* genes.

In potato it is difficult to determine *R* gene allelism by testcrosses. In the diploid cross *R3r* x *R6r* or *R3r* x *R7r*, no progeny was *R3R6* or *R3R7* (data not shown). To confirm the allelic relationship of the *R3*, *R5-R11* genes, we mapped several *R* genes from Group II and III in segregation populations using the anchoring CAPS marker GP250, which cosegregates with *R3a* and locates 0.4 cM proximal to *R3b* in SH (Huang et al., 2004). The Sc *R3*, Ma *R3*, *R6*, *R7*, and *R10* genes either

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Table 2. Marker analysis of the R3 allelic versions and control clones. Cultivars and clones are listed in the order of Table 1. Markers are listed according to their genetic order (Huang et al. 2004) and physical order (Huang et al., submitted) and are divided into two Sub-interval. Genotypes are ordered based on their similarity on marker patterns and are classified into three groups. R1089-039-16 and S1183-92-488 were used as references to define marker alleles (+ for absence and + for presence). The location of the St-12, R3a, and R3b clusters are marked on the right column.

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cosegregate with or reside 1-2 cM from GP250 (data not shown). We didn't map the MPI *R3*, FS *R3*, *R8* genes in Group II genotypes and the *R5* and *R11* genes in Group III genotypes, since their allelic relationship with the SH *R3* genes can be inferred from the mapping results of other genotypes in the corresponding groups.

To summarize, based on marker analysis (Table 2) and mapping results, we concluded that the *R3* clones with different pedigree and Ma *R5-R11* all contain allelic versions of the *R3* locus.

The functionally diverse *R3* allelic versions interact with *P. Infestans* in a gene-for-gene manner

To characterize the recognition specificities of the *R3* locus, we tested clones carrying different *R3* allelic versions with a panel of *P. infestans* isolates using a detached leaf assay or *in vitro* inoculation in duplicate experiments (Table 3A). Most interacting phenotypes observed at 5-7 days post inoculation (dpi) belonged to two types: localized hypersensitive (HR) necrosis that was classified as resistance (R) phenotype and intensive sporulation that was classified as susceptible (S) phenotype (Table 3A). Occasionally intermediate phenotypes were observed, as spreading HR necrotic lesion without sporulation classified as partial resistance phenotype (R*) and sporulation on part of inoculated leaflets or *in vitro* plantlets classified as partial susceptible phenotype (S*). These intermediate phenotypes were particularly associated with Ma *R10*, consistent with the statement that *R10* confers a rather weak resistance in contrast to other strong *R* genes, e.g. *R1* and *R3* (Vleeshouwers et al., 2000).

The allelic versions of the *R3* complex locus display a diversity of resistance specificities by interacting with the tested *P. infestans* isolates in a gene-for-gene manner (Table 3A). For example, *R5* was resistant to the isolate 90128 but susceptible to IPO-complex, and *R8* is *vice versa*. Ma *R3* was partially resistant to the isolate 89148-07, resistant to H30P04, and susceptible to 90206; while Sc *R3* was fully resistant to 89148-07, susceptible to H30P04, and partially resistant to 90206. Cv. Bintje was susceptible to all isolates. Except MPI *R3*, *R6* and *R7*, we distinguished all the resistance specificities using nine *P. infestans* isolates (Table 3A).

In brief, by phenotyping we identified nine allelic versions of the *R3* locus, i.e., SH *R3*, Ma *R3*, Sc *R3*, FS *R3*, *R5*, *R7*, *R8*, *R10*, and *R11*. It is not clear yet whether the *R3* allelic versions MPI *R3* and *R6* are functionally different from *R7*.

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Table 3. Resistance phenotypes and genotypes of the *R3* allelic versions.

A. Resistance phenotypes. Listed are allelic versions and cultivars/clones tested with nine *P. infestans* isolates (kindly provided by W. Flier of Plant Research International, Wageningen, the Netherlands and F. Govers of Laboratory of Phytopathology, Wageningen University, the Netherlands). S-susceptible, R-resistant, S*-partially susceptible, and R*-partially resistant.

B. Genotype of allelic versions shown by marker pattern (Table 2), haplotyping using PR3a and PR3b primers and sequencing. Different groups of marker pattern and different R3a and R3b haplotypes are displayed using color gradients. - no amplification, nd-no data.

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R gene sequence variation of the allelic versions of the *R3* complex locus

The functional diversity displayed by the allelic versions of the *R3* locus (Table 3A) is a contrast to the uniformity of marker patterns observed within each of Group II and III (Table 2). The variation of resistance specificities can be produced by means of gene conversion, intragenic unequal crossover, or diversifying selection (Michelmore and Meyers, 1998; Hulbert et al., 2001) without changing the flanking sequences. To determine whether the functional diversity can be reflected by the variation in *R* gene sequence, we characterized all the allelic versions with gene-specific primers for the *R3a* gene and the *R3b* gene candidate.

The P_{R3a} and P_{R3b} primers were designed from leucine rich repeat (LRR) domains of the *R3a* gene and the candidate *R3b* gene, *I2GA* (tomato *I2* gene analog)-*SH193-1*, respectively. This *I2*GA genetically cosegregates with the *R3b* gene in 3000 progeny of the *R3* mapping population and is homologous to the *R3a* and the tomato *Fusarium* wilt *I2* genes (data not shown). Unique single nucleotide polymorphisms (SNP) within the LRR domains of *R3a* and *I2GA-SH193-1* were identified from an alignment of all *I2*GAs found at the *R3* complex locus (**Chapter 4**; Huang et al., unpublished results) and were used to design the P_{R3a} and P_{R3b} primers. Gene-specific amplification in SH was confirmed using recombinants that contain *R3a* or *R3b* regions, SH bacterial artificial chromosome (BAC) clones carrying different *I2*GAs, BAC subclones carrying individual *I2*GAs, and sequencing (data not shown). Furthermore, the primers didn't amplify any fragment from genotypes in Group I (Table 1) and did amplify a single fragment of expected or slightly larger sizes from all Group II and III genotypes (data not shown). This confirmed that the *R3* allelic versions are not present in the Group I genotypes and also indicated that the amplified products from the Group II and III genotypes are derived from the *S. demissum* derived *R3* allelic versions instead of the *S. tuberosum r3* alleles or other genomic regions.

To investigate sequence variation conferred by both primers, the amplified fragments from some Group II and III genotypes were sequenced. The sequences displayed interesting variation among the *R3* allelic versions (Table 3B). Analysis of amplified sequences using the P_{R3a} primers led to the classification of the allelic versions into three haplotypes. SH *R3*, Sc *R3*, FS *R3*, MPI *R3*, Ma *R6a*, Ma *R7*, and Ma *R8* were identical and belonged to *R3a-haplotype-I*. Ma *R5* and Ma *R10* were identical and belonged to *R3a-haplotype-II* that had an insertion of 57 bp at the 3'end and shares 90% DNA identity with *R3a-haplotype-I*. Double peaks were observed in the sequencing trace file of *R3a-haplotype-II*, indicating that more than one homologue was amplified. Ma *R11* was the only member in *R3a-haplotype-III*,

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which also had a 57-bp insertion (different from the one in *haplotype II*) and shared 91% and 92% DNA identity with *R3a-haplotype-I* and *-III*, respectively. *R3a-haplotype-II* and *-III* are in-frame, suggesting they may be fragments of functional genes. The majority (80%) of SNPs between haplotypes resulted in amino acid substitutions and half of these substitutions are the predicted solvent-exposed residues of the LRR domain (data not shown). The sequences amplified by the P_{R3b} primers were also classified into three types (Table 3B) and were more than 96% identical to each other. SH *R3* and Ma *R3* belonged to *R3b-haplotype-I*; So *R3*, FS *R3*, MPI *R3*, Ma *R7*, and Ma *R8* fitted in *R3b-haplotype-II*; Ma *R10* and Ma *R11* were in *R3b-haplotype-III*. Sequences within each group are identical. *R3b-haplotypes-II* and *-III* were also in-frame, indicating that they were amplified from functional genes. There were 38 single nucleotide polymorphisms (SNPs) between *R3b-haplotypes-I* and *-III*. Thirty-four of these SNP resulted in 26 amino acid substitutions (Fig. 1). Sixteen of such substitutions were on the sites of the predicted solvent-exposed residues, which were recognized as the major determinants of recognition specificities of *R* genes. Interestingly, *R3b-haplotypes-II* appeared to be a recombinant of *R3b-haplotypes-I* and *-III* (Fig. 1), suggesting that intragenic recombination was involved in producing new recognition specificities at the *R3b* region.

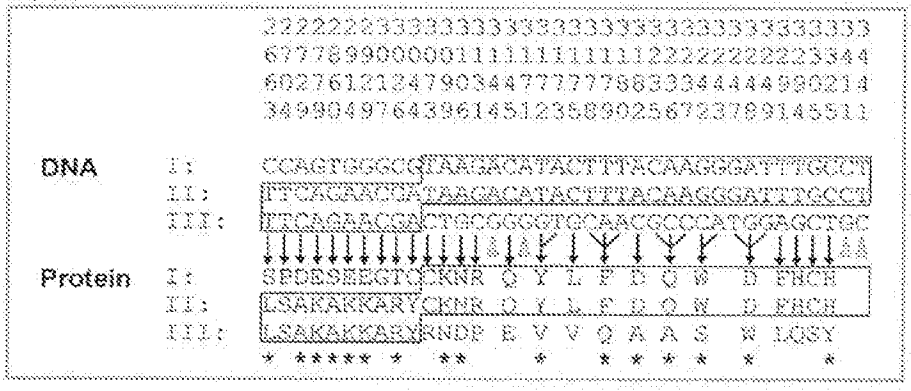


Fig. 1. Allelic variation of the *R3b*-candidate haplotypes (I-III) at both DNA and protein levels. Display of all 38 SNPs. The position of each nucleotide site relative to the first nucleotide of the ATG start codon of the full *R3b*-candidate gene is given by vertical numbers above each site. The DNA and or protein sequences shared by two haplotypes were contained in the brackets. All SNPs involved in amino acid substitutions are connected to the corresponding residues by arrows. Synonymous substitutions are marked with grey triangles. Putative solvent-exposed residues are predicted based on the *R3b* candidate genes and are marked by asteroids.

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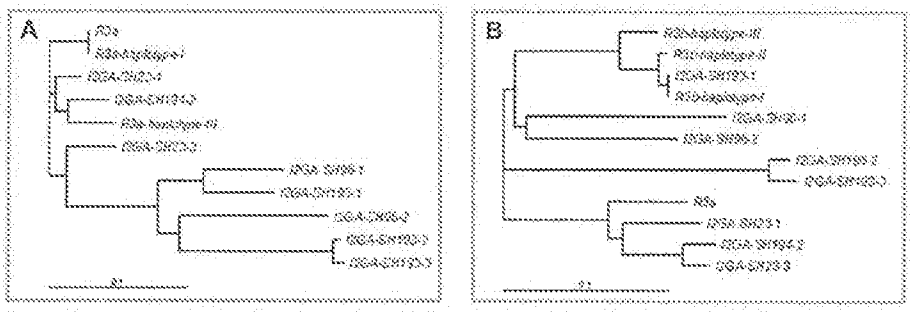


Fig. 2. Distance trees of *R3a*- (A) and *R3b*- (B) haplotypes with *I2*GAs at the *R3a* and *R3b* clusters in SH. Four *I2*GAs (*R3a*, *I2GA-SH23-1*, *-3*, and *I2GA-SH194-2*a) are from the *R3a* cluster and five *I2*GAs (*I2GA-SH96-1*, *-2*, *I2GA-SH193-1* (the *R3b* candidate), *-2*, and *-3*) are from the *R3b* clusters. Sequences at the window defined by the P_{R3a} and P_{R3b} primers in the nine *I2*GAs were extracted and aligned with the *R3a*-haplotypes (I and III) and *R3a*-haplotypes (I, II, and III), respectively. *R3a-haplotype-II* was excluded from the analysis since it contains double peaks in the sequencing trace file. The trees was calculated using Clustal X (Thompson et al., 1997) and visualized using Treeview (Page, 1996).

It remains to be confirmed that allelic/orthologous relationships exist between the amplified sequences by each pair of primers. However, sequence similarity analysis revealed that *R3a*-haplotypes were more similar to *I2*GAs in the *R3a* cluster than those in the *R3b* cluster but displayed an obscure allelic/orthologous relationship with the *I2*GAs within the SH *R3a* cluster (Fig. 2A). *R3a-haplotype-III* is more similar to *I2GA-SH194-2* rather than the *R3a* gene. The *R3b*-haplotypes are more similar to the candidate *R3b* gene-*I2GA-SH193-1* than any other paralogs in SH (Fig. 2B), suggesting their allelic/orthologous relationships. It remains unclear whether these sequence variations are involved in late blight resistance specificities among the *R3* allelic versions. However, the classification of *R3a*- and *R3b*-haplotypes quite fitted our observation of resistance phenotypes (Table 3). First, the *R3a* phenotype (resistant to *P. infestans* isolates 89148-09, IPO-0, 89148-07 and susceptible to H30P04) was closely associated with *R3a*-haplotype I and the marker pattern displayed in Table 2, indicating the presence of *R3a* in these allelic versions. The resistance of Ma *R8* to the isolate H30P04 may be conferred by another functional gene. Second, the response to the isolate H30P04 was associated with *R3b*-haplotypes I and II. SH and *R5* were resistant to H30P04 and were classified into *R3b*-candidate haplotype I. All genotypes in the second haplotype are susceptible to H30P04, except Ma *R8* that could be explained by the reasons mentioned above. Third, *R10* and *R11* had the same *R3b*-candidate-haplotypes, which is consistent with the observation that the corresponding *Avr10* and *Avr11* genes are associated (van der Lee et al., 2001b).

The linkage disequilibrium among the *R3* allelic version included the *St-I2* cluster (Table 2), suggesting that functional late blight *R* genes at the cluster may be co-selected with the *R* genes at the *R3a* and *R3b* clusters during introgression. This

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might explain the phenotypic difference (Table 3) between the allelic versions that carry *R3a*-haplotype I and *R3b*-candidate haplotype II. The recombination between the three clusters may be a potential mechanism to create new resistance specificities. For example, *R5* could be regarded as a recombinant between SH *R3* and *R10*, and *R10* as a recombinant between *R5* and *R11*.

In summary, the *R* gene variation revealed by the P_{R3a} and P_{R3b} gene-specific primers correlated with resistance specificities displayed by differential *P. infestans* isolates and thus can be used as an indicator of allelic phenotype variation. Genetic reshuffling between the *St-I2*, *R3a*, and *R3b* clusters within the complex *R3* locus likely plays an important role in generating new resistance specificities by the *R3* allelic versions.

Discussion

In this report we revisit the history of 150 years in potato resistance breeding against its most devastating disease late blight. We report the unexpected discovery that the majority of characterized resistance specificities introgressed from *S. demissum* contain allelic versions of the *R3* complex locus. We also suggest that diversifying selection, intragenic recombination, and reshuffling between the three clusters within the complex *R3* locus are potential mechanisms involved in the generation of these allelic versions. This study may have two important implications.

First, breeding against *P. infestans* stopped too soon! Despite over a century's effort in utilizing *R* genes for late blight control, the potential of late blight resistance breeding is still under exploration. Our discovery demonstrated that only four *R* loci (*R1-4*) were explored from the main resistance source *S. demissum* and only a very limited *S. demissum* accessions (less than 10 as described in Table 1) were used. From the 11 resistance specificities characterized (*R1-11*), only *R1-3* and *R10* have been used in commercial potato production. *R8* that displayed a high field resistance (Haynes et al., 2002) has never been used in breeding, which now can be explained by the allelic relationship of *R3* and *R5-11*. If a tetraploid cv. already contains *R3* and *R10*, combining a third *S. demissum* allele might greatly influence the vigour of the cultivar as at diploid level the plants with two *R3* allelic versions are lethal (A. El Kharbotly and E. Jacobsen, unpublished results). The accumulating knowledge on the *R3* complex locus (**Chapter 4**; H. Kuang and S. Huang, *unpublished results*) will enable cloning of its multiple allelic versions in the near future. Using GMO approaches, breeders can use these allelic genes in elite potato cvs. by means of pyramiding or polyculture or a combination of both. This message should at least ease the disappointed atmosphere circling among the potato breeders who have adopted the *R*-gene free strategy.

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To pursue a single gene with durable resistance is appreciable but might be impossible. Race specificity of late blight *R* genes was not unique to *S. demissum* but rather a general phenomenon, since it was also reported in *S. pinnatisectum*, *polyadenium*, *stoloniferum*, and *verrocosum* (Black and Gallegly, 1957; Rivera-Pena, 1990d, V.G.A.A. Vleeshouwers, personal communication). The race specificity of *R* genes in the hosts can be well explained by the high evolutionary potential of the pathogen (McDonald and Linde, 2002). The recently cloned broad-spectrum *RB/Rpi-blb1* gene might recognize the "Achilles heel" of the pathogen. This seems to offer a new hope to for *R* gene monoculture in potato. However, we noticed that *S. bulbocastanum* often grows in dry conditions and locates in lower altitude range (1,500-2,300m) than *S. demissum* (2,650-3,800m, Hawkes, 1990), which was often found on the cool mountain-forests in the Toluca Valley of Mexico. The putative lacking of co-evolutionary history between *S. bulbocastanum* and *P. infestans* could mean that the pathogen has not unleashed its great mutation potential (McDonald and Linde, 2002) to overcome genes like *RB/Rpi-blb1* in the host. The "boom-bust" cycles can be repeated on deployment of *RB/Rpi-blb1* as it already happened to *R3*.

Second, the allelic diversity of the *R3* complex locus implied that *R* gene polyculture (multiline) could be a potential mechanism adopted by *S. demissum*. Detailed investigations were conducted by Rivera-Pena (1989; 1990a-d) on wild populations of *S. demissum* at the Toluca Valley, which was recognized as a diversity centre of *P. infestans*. Several of his findings are quite interesting: the disease incidence on wild populations is low; the *R3* allelic versions (*R3*, *R6-8*, *R10*, and *R11*) are resistant to 35-78% of the local isolates; all *S. demissum* plants collected are resistant to race 0 and 67% show a clear HR, indicating the presence of major *R* genes, while 85% of these *S. demissum* plants are susceptible to an aggressive complex isolate. Although other factors such as small population sizes and mixtures with nonhosts undoubtedly contribute to the durable resistance of wild *S. demissum* populations in the Toluca Valley, we think that the recognition heterozygocity at the population level conferred by the *R3* locus and possibly others is an important factor toward durability. Population genetics predicts that the polymorphism of parasite recognition capacity in a host population will make that most isolates of the parasite can not grow on most hosts (Hamilton et al., 1990).

We suggest that *R* gene polyculture is a better approach than pyramiding in late blight control. Several mechanisms have been postulated to explain the reduction in severity of disease in *R* gene polyculture (Jones, 2001; Mundt, 2002). One mechanism is the fitness penalty on the pathogen due to loss of avirulence. As early as in 1952, Bill Black, one of the pioneers in late blight research, already reported the first evidence of fitness drawback of virulence (Black, 1952). He

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reported that the isolates that overcame *S. demissum R* genes could not compete with race 0 on susceptible plants. In addition, although fully complex isolates have been reported, they are not frequently found in potato fields (Stewart and Bradshaw, 2001). Another mechanism is the dilution of inoculum that occurs due to increased distance between plants of the same genotype. Late blight disease development on detached leaflets (Stewart, 1990) and *in vitro* plantlets (Huang et al., unpublished data) of potato was influenced by inoculum concentration of *P. infestans*. On the other side, *R* gene pyramiding doesn't virtually differ with monoculture in the aspect of recognition uniformity, which is vulnerable to highly evolutionary risky pathogens such as *P. infestans*. Imagine that we create a super potato by pyramiding all the cloned genes by GMO approach or conventional breeding and grow it in a large acreage, this would put an enormous pressure to the pathogen. By virtue of its overwhelming advantage in rate of evolution (McDonald and Linde, 2002), *P. infestans* will eventually mutate all the corresponding avirulence genes and have the upper hand against the host. Another risk of pyramiding is the fitness cost of *R* gene to the host, which was for the first time elegantly demonstrated on the Arabidopsis *RPM1* gene (Tian et al., 2003). There are also some hints about the cost of late blight *R* genes. For example, segregating populations often skew toward susceptibility (Müller and Black, 1952; El Kharbotly et al., 1994). From an ecological viewpoint, our philosophy of late blight disease management is not to eradicate the pathogen but maintain equilibrium between the pathogen and the host by using the combination of *R* gene polyculture, cultivation measures, and limited application of pesticide.

Diseases have a great impact on the evolution of interacting host, which acts on the locus responsible for recognition (Howard, 1991). Allelism is an efficient way of creating recognition polymorphism (Bergelson et al., 2001). The most striking example is the vertebrate major histocompatibility complex (MHC) that plays a key role in the immune system by presenting peptides to T cells and has an extraordinarily high levels of polymorphism (Hughes and Yeager, 1998). The MHC polymorphisms are characterized by a large number of alleles differentiated mainly at the peptide binding domain. An analogy was found at the *R3* complex locus, the at least nine allelic versions were derived from only five to seven *S. demissum* accessions (Table 1). The accession CPC2127 contains three differential allelic versions, Sc *R3*, *R7*, and *R8*. Most of the SNPs (34/38) between *R3b*-candidate haplotypes resulted in amino acid changes (Fig. 1), indicating that significant diversifying selection was involved in the evolution of the locus. Further, 62% (16/26) of the substitutions are the predicted solvent-exposed residues in the LRR domain that are regarded as the main determinants of *R* gene recognition

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specificities (Parniske et al., 1997; Van der Hoorn et al., 2001; Wulff et al., 2001), suggesting that natural selection resulted in creating new resistance specificities in the *R3* locus. Most complex *R* loci contain only a single *R* gene with known function (Hulbert et al., 2001). The *R3* complex locus consists of three clusters and in two of these clusters functional *R* genes have been found. The genetic reshuffling between the clusters could be an important mechanism in generating high allelic variation of the *R3* complex locus (Table 3). Suppose if each of the St-I2, *R3a* and *R3b* clusters has 10 alleles, then recombination between the two clusters can create a maximum of 1000 allelic versions for the locus. These observations, together with the discovery of the physical expansion of the locus compared to the syntenic region in tomato (**Chapter 4**), led to a plausible presumption, that is, it is not an incidence that the majority of known *S. demissum* resistance specificities carry allelic versions of the *R3* locus. Instead, we believe the locus plays a general role in *S. demissum* to control the fast-evolving *P. infestans*. We therefore designate it as the major potato late blight resistance locus in *S. demissum*. The cloning of its allelic versions and subsequent deploying them in elite cultivars via *R* gene polyculture will enable breeders to mimic the tricks of *S. demissum* for late blight control in potato field.

Materials and Methods

Plant materials

All plant materials are listed in Table 1. Their pedigree information is maintained in the online database (http://www.dpw.wur.nl/pv).

Marker analysis

DNA isolation, AFLP and CAPS marker analysis were described previously (Huang et al., 2004). All marker alleles have been examined in duplicate experiment and scored independently by two persons.

Resistance phenotyping

Maintenance of *P. infestans* isolates, preparation of inoculum were described previously (Vleeshouwers et al., 1999). Resistance was scored on detached leaves (van der Lee et al., 2001a) and in vitro plantlets (Huang et al., *submitted*). At least two duplicate experiments were conducted to determine the interacting phenotype of each genotype with each isolate.

R3a and *R3b*-candidate haplotyping

Using CLUSTAL X 1.81(Thompson et al., 1997), we created a DNA sequence alignment of *R3a, I2GA-SH23-1, -3*, and *I2GA-SH194-2* from the *R3a* cluster (Huang et al., *submitted*) and *I2GA-SH96-1, -2, I2GA-SH193-1* (the *R3b* candidate), *-2*, and *-3* from the *R3b* cluster (Huang et al., unpublished work). Gene-specific primers P_{R3a} (F: ATCGTTGTCATGCTATGAGATTGTT R: CTTCAAGGTAG -TGGGCAGTATGCTT) and P_{R3b} (F: GTACTAGA-GGAACTTTTGATTTATTG R: GACTGAATTT -GAGGC AACTCGC) were designed on the sequences of *R3a* and *I2GA-SH193-1* by localizing gene-specific SNPs at the 3' end of the primers. Touchdown PCR (68-54 °C and 60-50 °C for the P_{R3a} and P_{R3b} primers, respectively) was applied to ensure primer specificities. PCR product was purified

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using Qiaquick kits of Qiagen and sequenced with gene-specific primers from both ends.

To investigate the possible allelic/orthologous relationship between the amplified *R3a*- and *R3b-haplotypes* with the four *I2*GAs in the *R3a* cluster and the five *I2*GAs in the *R3b* clusters of SH, sequences of the nine I2GAs at the P_{R3a} and P_{R3b} windows were extracted and alignment with sequences *R3a*- and *R3b*-haplotypes. *R3a-haplotype-II* was excluded for this analysis since it has double peaks in the sequencing trace file. The alignments were created by CLUSTAL X 1.81 (Thompson et al., 1997) and distance tree was calculated using the neighbour-joining method. Positions with gap were excluded for calculation.

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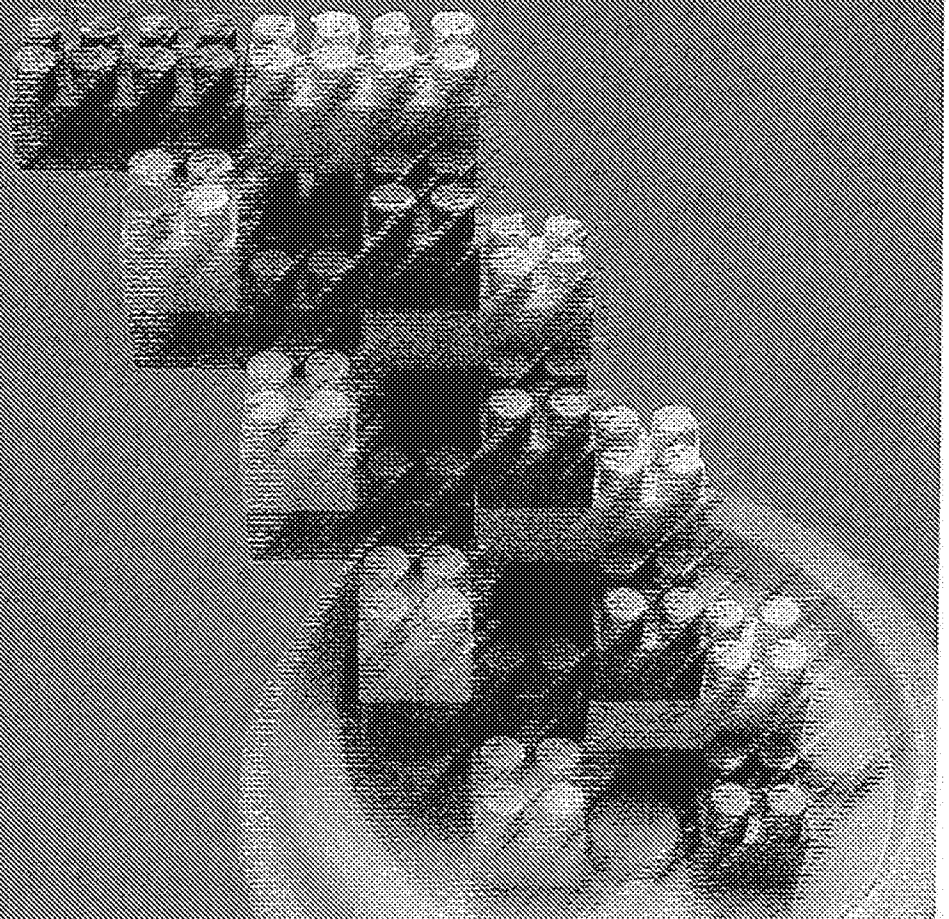
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Chapter 6

General Discussion



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