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

genomic structure, functional diversity, and implications

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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].

	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	6.7	3.4	2.8	5
Netherlands	7,226	165	43.8	0.9	0.9	0.0	1
World	318,138	19,388	16.4	0.4	0.1	0.3	4

	Utilization					Con-sump.	Trade [c]			
	Food	Feed	Seed	Process.	Other	per capita	Imports		Exports	
			%			kg/yr	x1000 t	M$	x1000 t	M$
China	36	31	6	22	5	14	3.7	4	79.4	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 importantce 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

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.

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

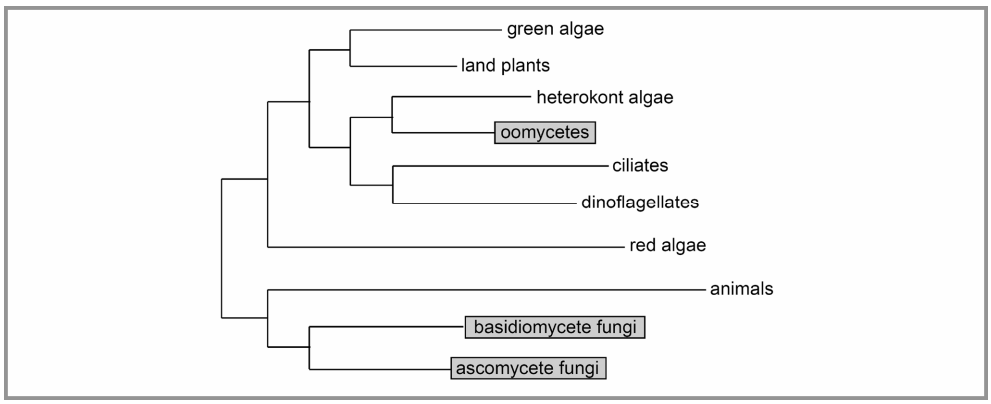
Phytophthora infestans (Mont.) de Bary is the cause of potato late blight and is also known as the Irish potato famine fungus. In Latin, *Phytophthora* means plant destroyer, which exactly fits this notorious pathogen. Late blight totally destroyed Ireland's potato crop during 1845 and 1846, which resulted 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

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

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

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.

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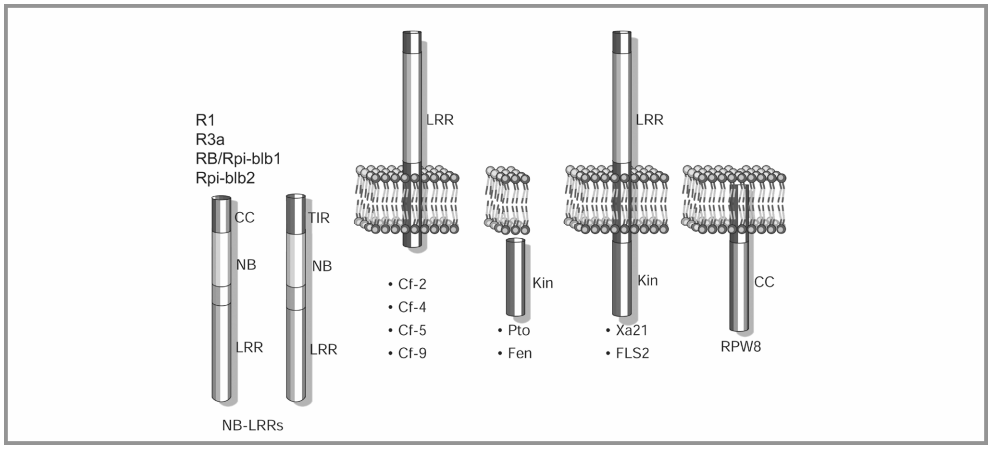

Fig. 2. Representation of the cellular location and structure of the five main classes of plant disease resistance proteins. Xa21 and Cf-X proteins carry transmembrane domains and extracellular LRRs. The RPW8 gene product carries a putative signal anchor at the N terminus. The Pto gene encodes a cytoplasmic 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

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)

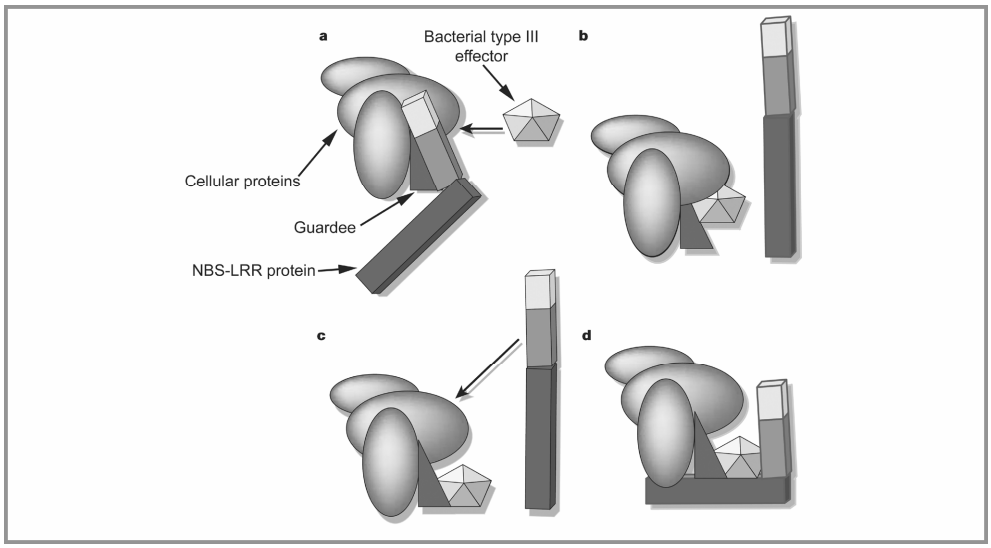

Fig. 3. Scenarios for the guard hypothesis for R-protein function. Reprod. from Dangl&Jones (2001).
a, A cellular complex of proteins, which includes both the 'guardee' molecule and an NBS-LRR protein (grey, shaded from the N terminus through NBS and LRR domains), is a target for a bacterial type III 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.

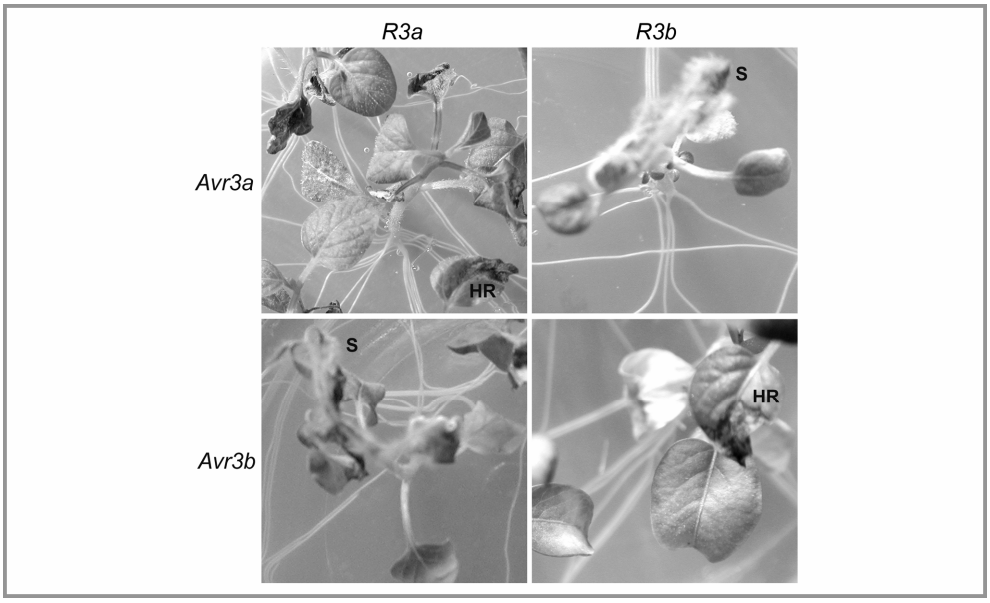

Fig. 4. The gene-for-gene interaction between potato and *P. infestans*. Phenotype of plants carrying *R3a* and *R3b* inoculated with *P. infestans* isolates carrying *Avr3a* and *Avr3b*. Localized hypersensitive necrosis (HR) and sporulating lesions (S) are observed on the incompatible and compatible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

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. berthautii*, *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

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

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. okade*	9	Unclear	
Rpi-oka2		4	Unclear	

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

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-

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

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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Chapter 2

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

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Submitted

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

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Richard G.F. Visser, Evert Jacobsen

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

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

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 4642-1
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 2424 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

2.5 x 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.

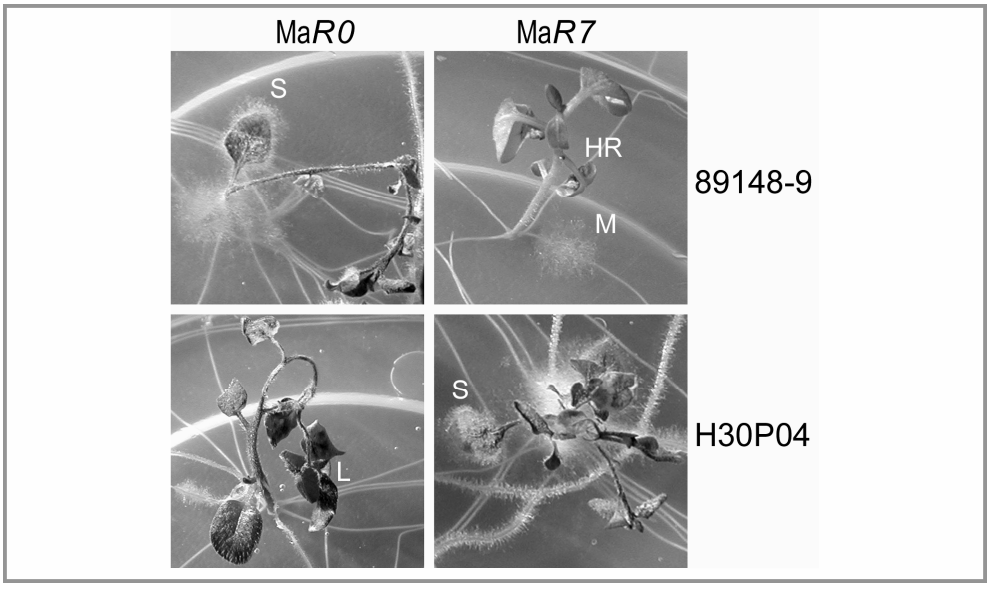

Fig 1. Compatible and incompatible interaction observed on *in vitro* plantlets. The *in vitro* plantlets of Ma*R0* and Ma*R7* inoculated with *P. infestans* isolates 89148-9 (race 0) and H30P04 (race 3a.7) at six dpi. A race-specific resistance response occurred on Ma*R7* leaves upon inoculation with 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.

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**
Ma*R0*	S (1)	S (1)	S (1)	S (1)	S (1)	S (1)	S (1)	S (1)
Ma*R1*	S (1)	S (1)	S (1)	S (1)	R (5)	R (5)	R (5)	R (5)
Ma*R2*	R (5)	R (5)	R (5)	R (5)	R (5)	R (5)	R (5)	R (5)
Ma*R3*	R (5)	R (5)	R (5)	R (4)	R (5)	R (5)	S (1)	S (1)
Ma*R4*	R (5)	R (5)	S (1)	S (1)	R (5)	R (5)	R (5)	R (5)
Ma*R5*	R (5)	R (5)	R (5)	R (5)	R (5)	R (5)	R (5)	R (5)
Ma*R6*	R (5)	R (5)	R (5)	R (5)	R (5)	R (5)	R (5)	R (5)
Ma*R7*	R (5)	R (5)	R (5)	R (4)	S (2)	S (1)	S (1)	S (1)
Ma*R8*	R (5)	R (5)	R (5)	R (5)	R (5)	R (5)	R (5)	R (5)
Ma*R9*	R (5)	R (5)	R (5)	R (5)	R (5)	R (5)	R (5)	R (5)
Ma*R10*	R (5)	R (5)	R (5)	R (4)	R (4)	R (5)	R (5)	R (5)
Ma*R11*	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 $\leq$ 2.0),

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

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

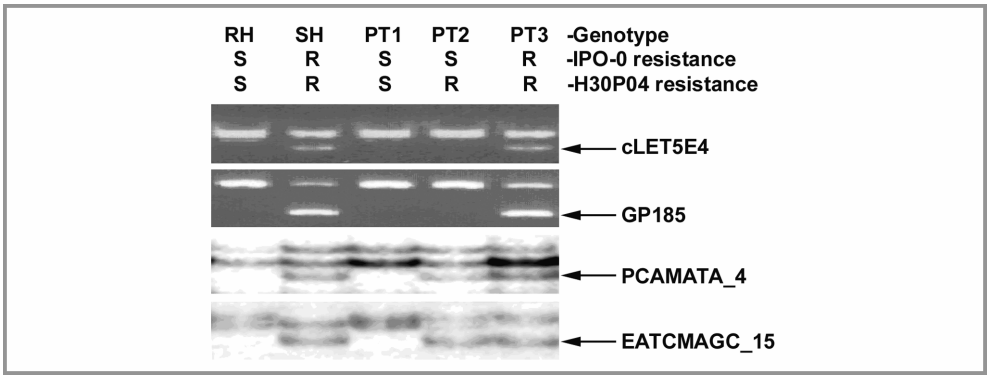

Fig 2. Genotyping of the three progeny types (PT) in the SH x RH *R3* mapping population and the parental clones RH and SH with four molecular markers. cLET5E4 and GP185 are flanking *R3a* and PCAMATA_4 and EATCMAGC_15 are flanking *R3b*. The markers are indicated at the right with an arrow pointed to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

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 demonstrated 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

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

described (Vleeshouwers et al., 1999). For each *in vitro* plantlet, the three largest leaves were inoculated with a zoospore suspension of 2.5 x 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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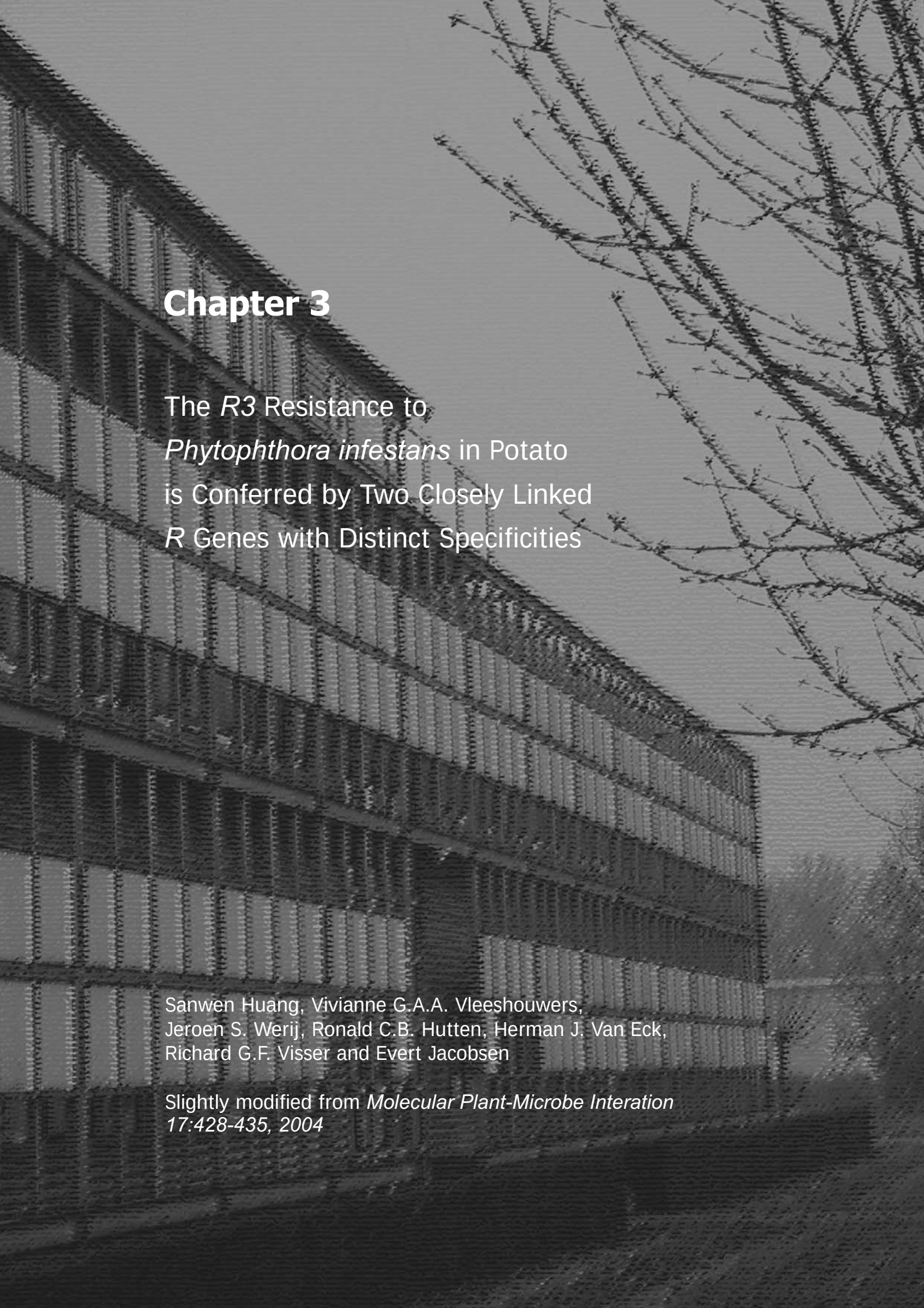
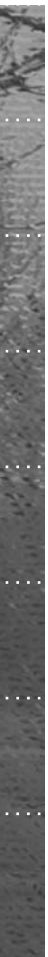


Chapter 3

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

Sanwen Huang, Vivianne G.A.A. Vleeshouwers, Jeroen S. Werij, Ronald C.B. Hutten, Herman J. Van Eck, Richard G.F. Visser and Evert Jacobsen

Slightly modified from *Molecular Plant-Microbe Interation 17:428-435, 2004*



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

Sanwen Huang, Vivianne G.A.A. Vleeshouwers, Jeroen S. Werij,
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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

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

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
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* (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

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	60-52 [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
TG26	65/66	C	GAG AGG GGA CAC TTT TAT TTA TTC A GAG ATC TTC CCG CCG CTG TG	49	1600	a.s. [b]
CT120	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

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 *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 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 Univerisity, The Netherlands
IPO-0	0	3b	W. Flier, Plant Research International, The Netherlands
H30P04	7	3a.7	F. Govers, Wageningen Univerisity, The Netherlands
89148-27	3.7	3a.3b.7	
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

of a single dominant *R* gene (χ^2 = 2.37; p≥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*.

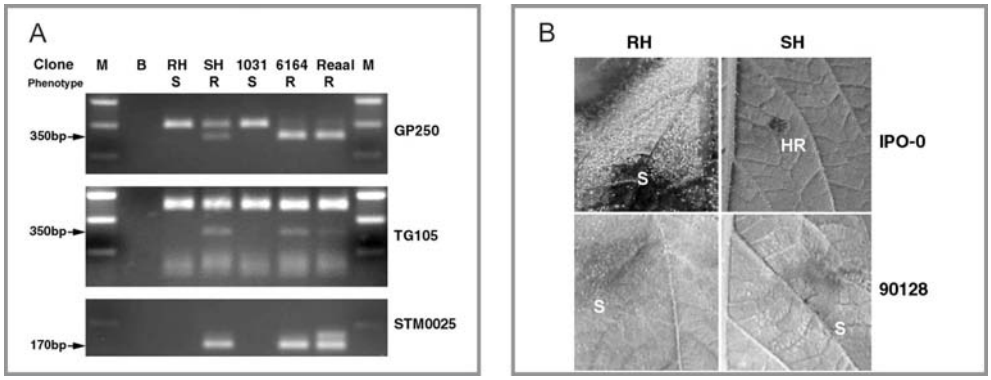

Fig. 1. Molecular and biological evidence for the presence of *R3* in SH.
A. Analysis of the genomic DNA from RH, SH, 1031, 6164, and Reaal with markers GP250 (*Vsp*I), TG105 (*Hin*fI), and STM0025. The phenotype (R-resistant, S-susceptible) of these clones inoculated with *P. infestans* isolate IPO-0 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

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.

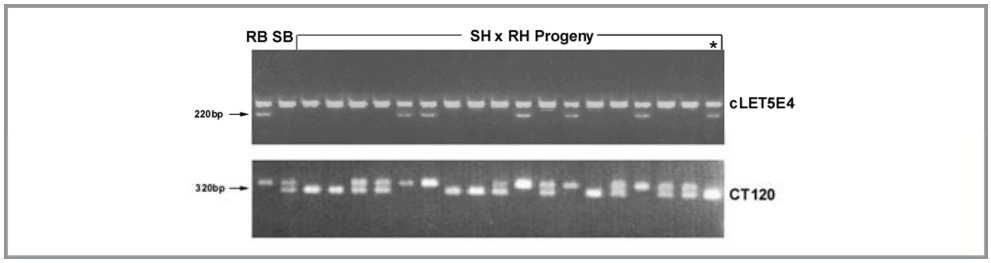

Fig. 2. Screening for recombination events near the *R3* locus in the SH x RH progeny with two flanking markers, cLET5E4 (*Hha*I) and CT120 (*Tsp*509I). The sizes of the diagnostic bands are indicated on the left. RB, resistant bulk consisting of 20 resistant plants; SB, susceptible bulk consisting of 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,

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.

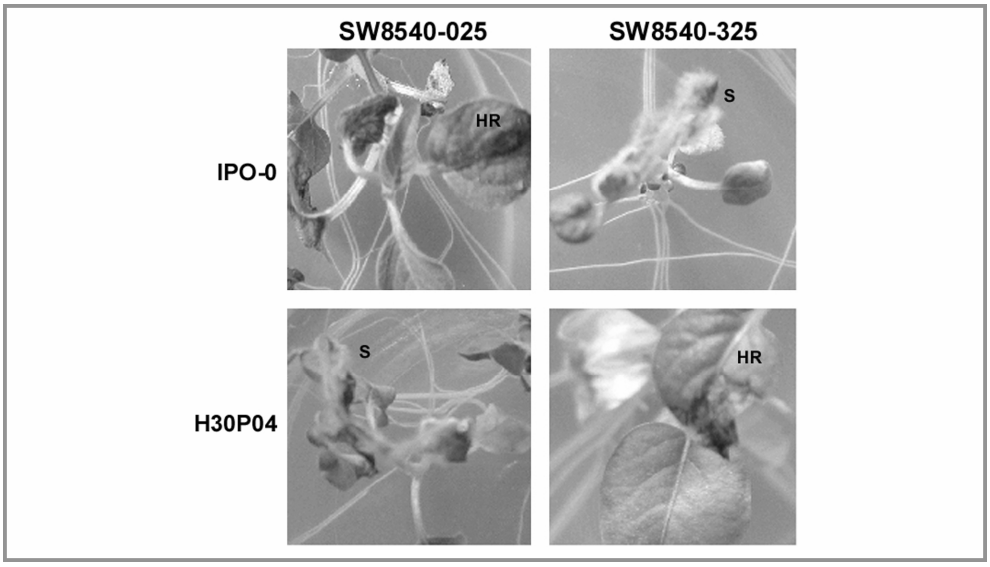

Fig. 3. Phenotype of differential resistance response at 6 dpi with *P. infestans* isolates IPO-0 and H30P04 on *in vitro* plants. SW8540-025 is resistant (HR) to IPO-0 and is susceptible to H30P04 as shown by the sporulating lesions (S). SW8540-325 shows the opposite response.

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.

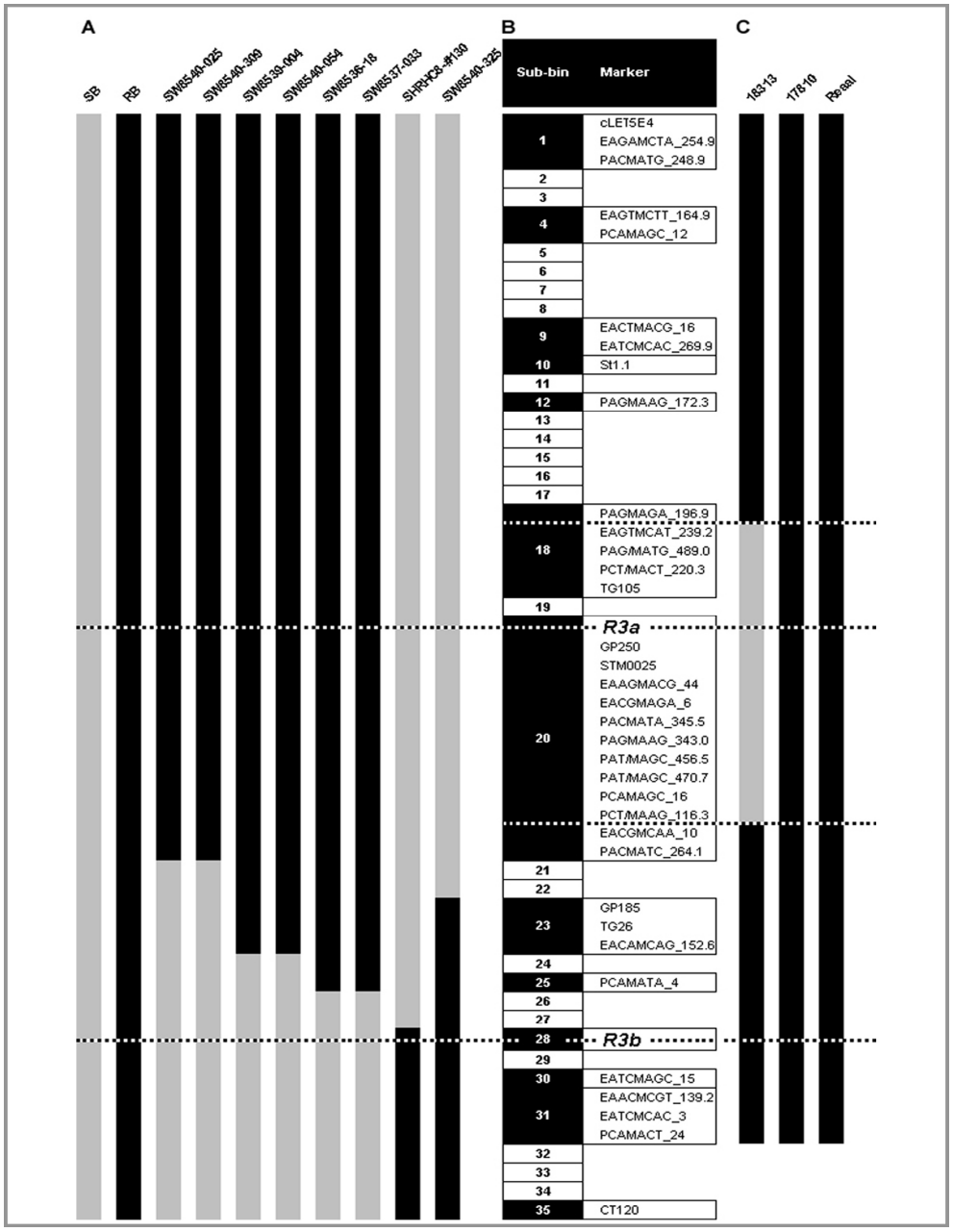

Fig. 4. High-resolution genetic map of *R3a* and *R3b*.

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.

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

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.

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 Martinez-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

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 *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.

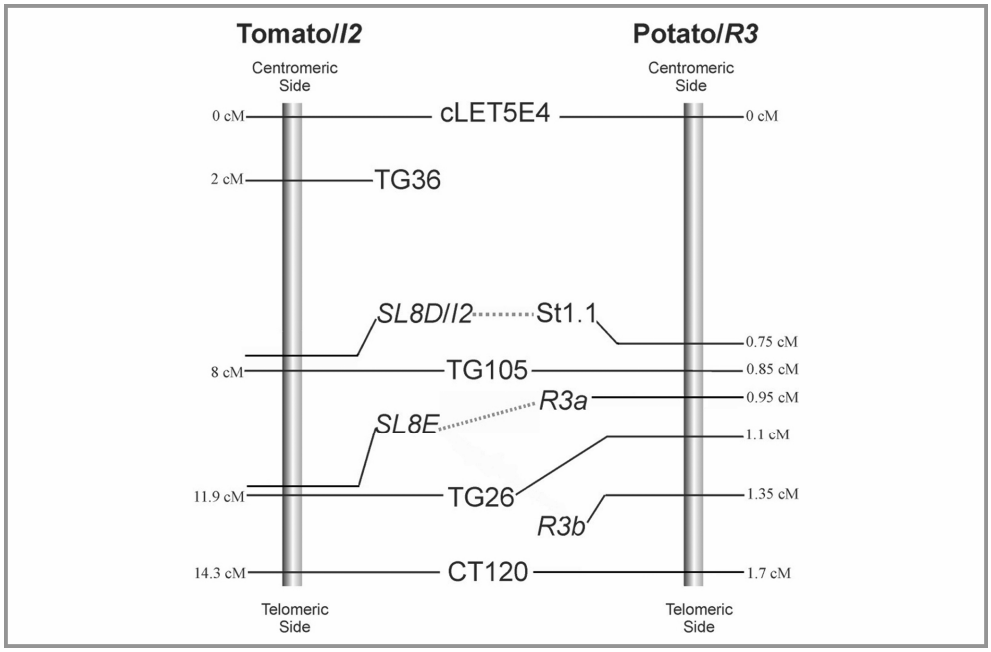

Fig 5. The synteny between the *R3* locus on the short arm of chromosome 11 in potato clone SH and the *I2* locus on the long arm of chromosome 11 of tomato. The left and right solid bars represent the tomato and potato genome, respectively. Mapped markers or genes are connected by black lines. On the 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

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

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 (cLET5E4, 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 94ºC, 30 seconds annealing at Tm (Table 2) and 45 seconds elongation at 72ºC. As a first step in PCR-amplification DNA was denatured for five min at 94ºC and finalized by an extra seven min elongation step at 72ºC. 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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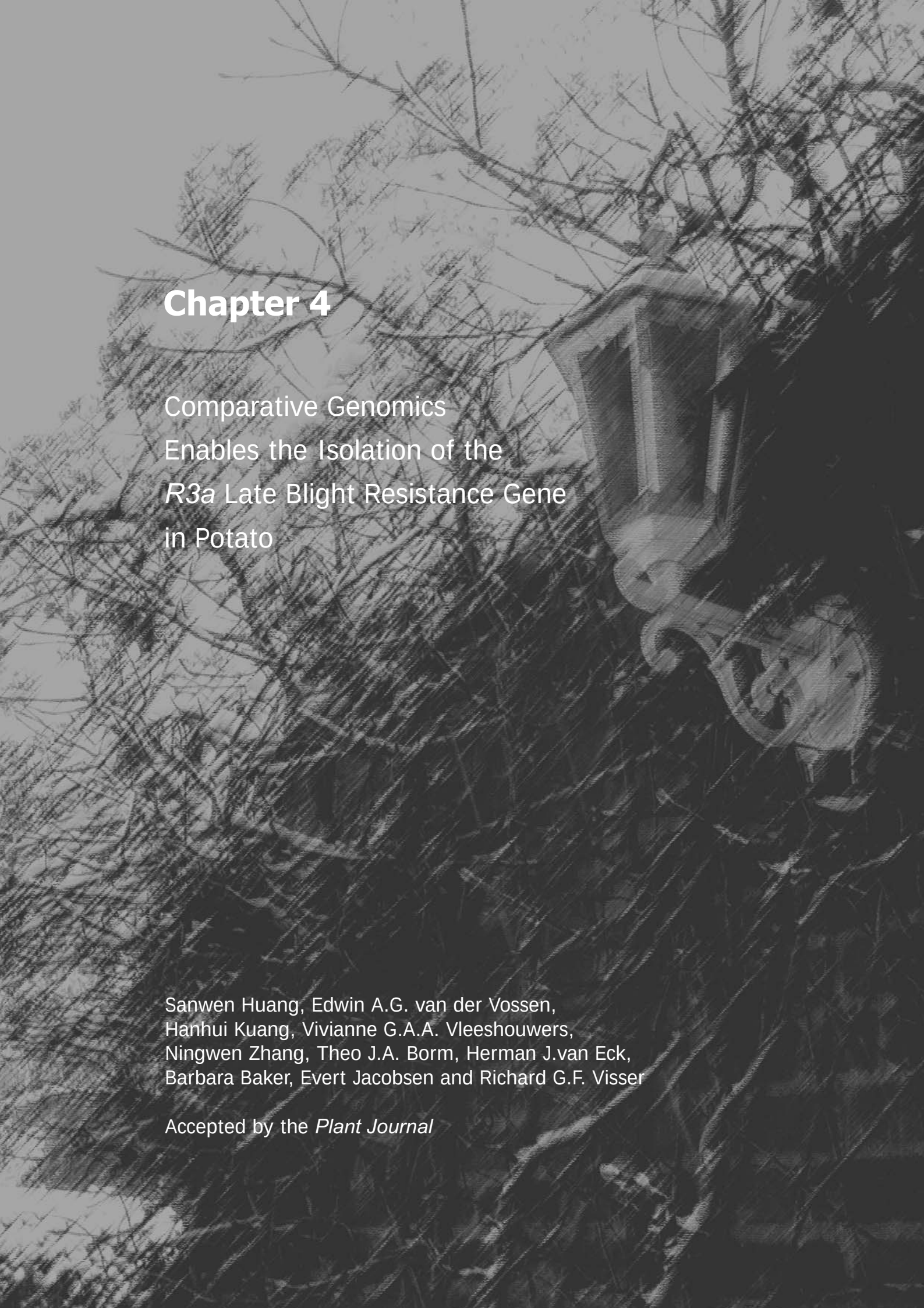

Chapter 4

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

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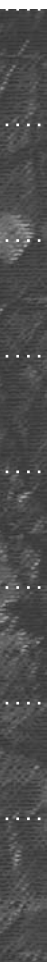

Comparative genomics enabled the isolation of the *R3a* late blight resistance gene in potato

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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

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

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

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.

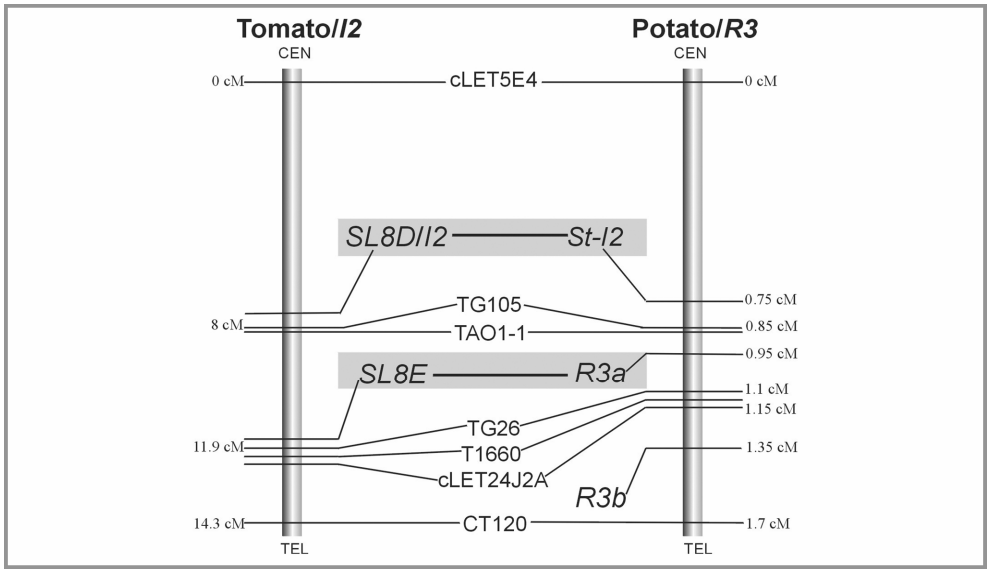


Fig. 1. Comparative genetic maps of the *I2* complex locus in tomato and the *R3* complex locus in potato. The left and right solid bars represent the tomato and potato chromosomes, respectively. Orientation is indicated by CEN (centromere) and TEL (telomere). Mapped markers are connected by black lines. 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 *St-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.

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

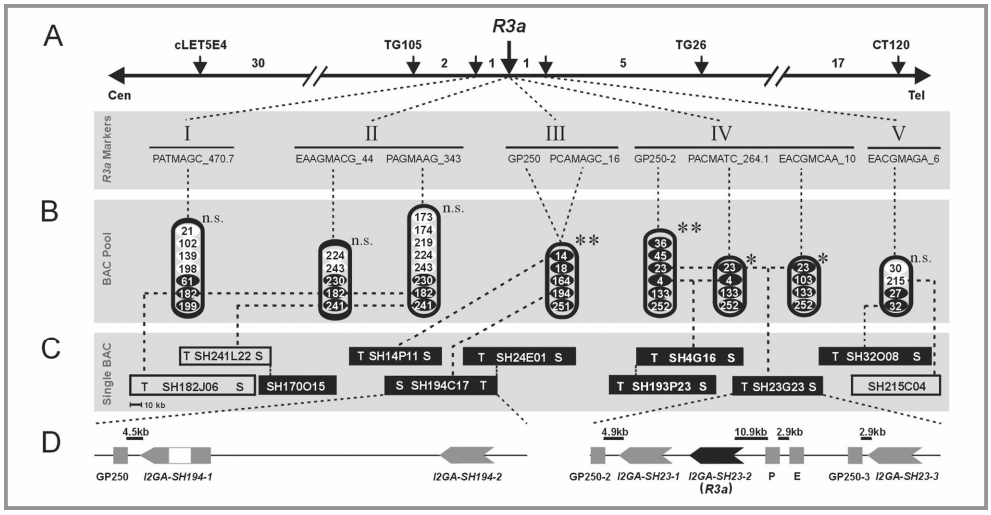


Fig. 2. Genetic and physical mapping of *R3a*.

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_{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 (Fig. 2A and 2B). 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 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
	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 Lab of Phytopathology, Wageningen University and IPO-0 by Dr. W. Flier of Plant Research International, Wageningen, the Netherlands.

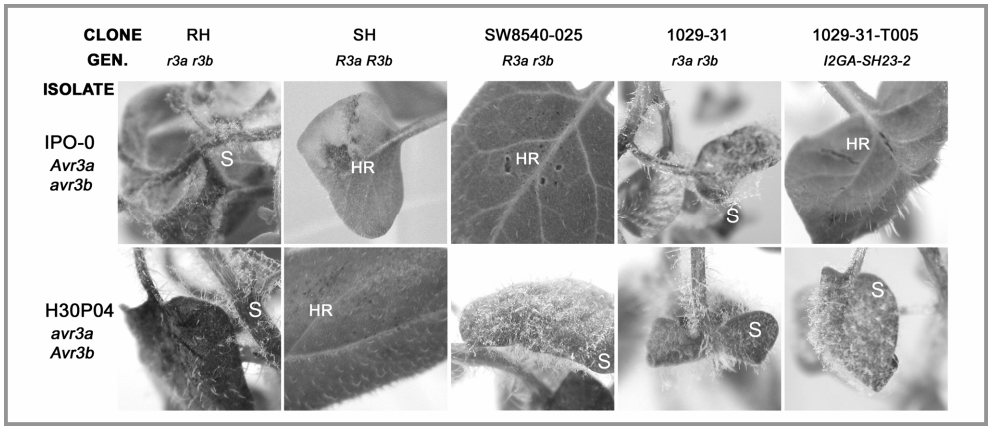


Fig. 3. *In vitro* inoculation of the primary transformants of *R3a*. Massive sporulation (S) and localized hypersensitive reactions (HR) are observed on compatible and incompatible interactions, respectively. RH and 1029-31 are susceptible to both isolates. SH is resistant to both isolates. SW8540-025 (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).

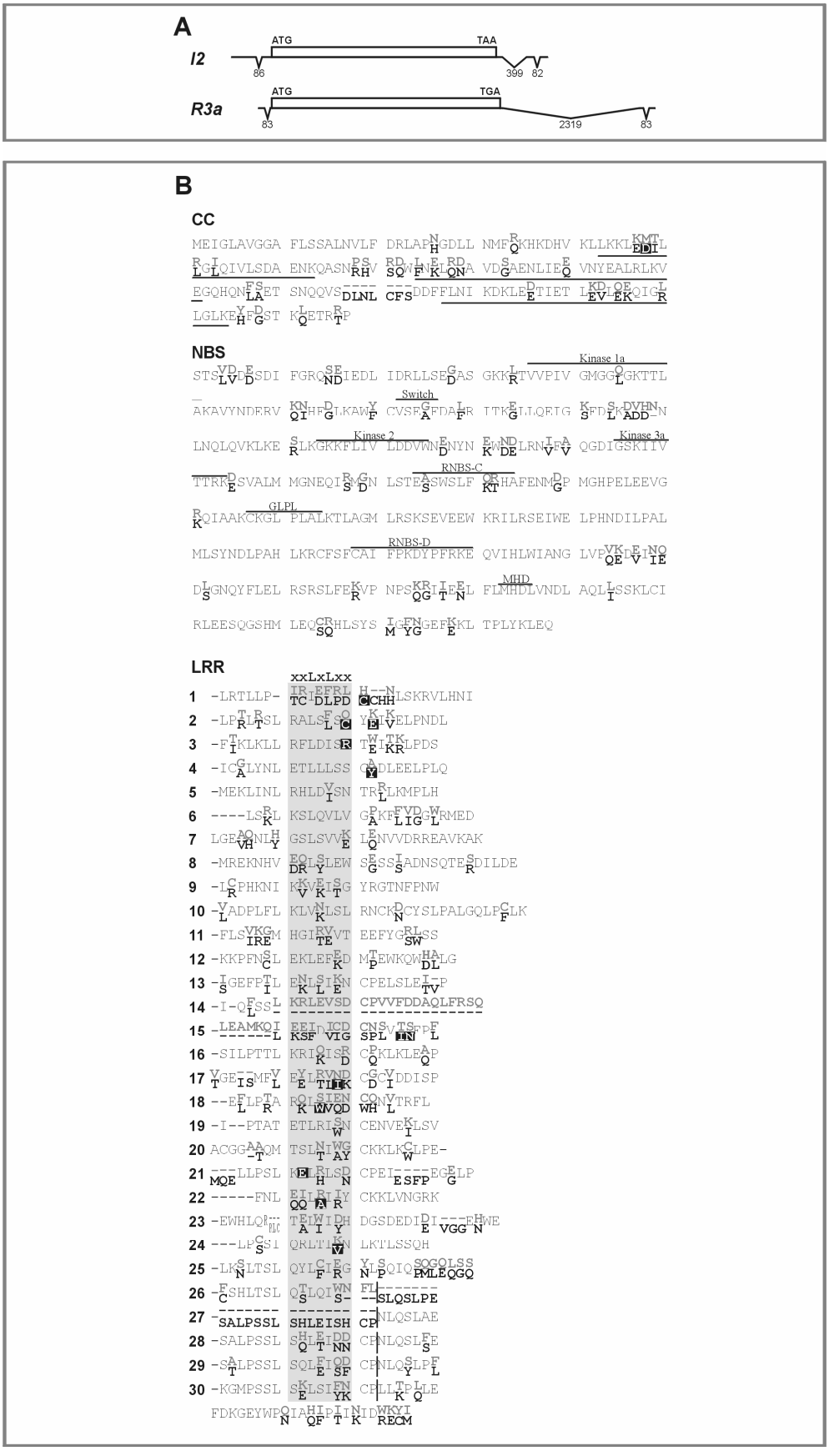
The *R3a* gene encodes a putative CC-NBS-LRR protein and shares 88% DNA identity and 83% amino acid (a.a.) similarity to *I2* (Fig. 4B). Considerable

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.


A
I2
ATG
TAA
86
399
82
R3a
ATG
TGA
83
2319
83
B
CC
NBS
Kinase 1a
Switch
Kinase 2
Kinase 3a
RNBS-C
GLPL
RNBS-D
MHD
LRR
xxLxLxx

← **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

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.

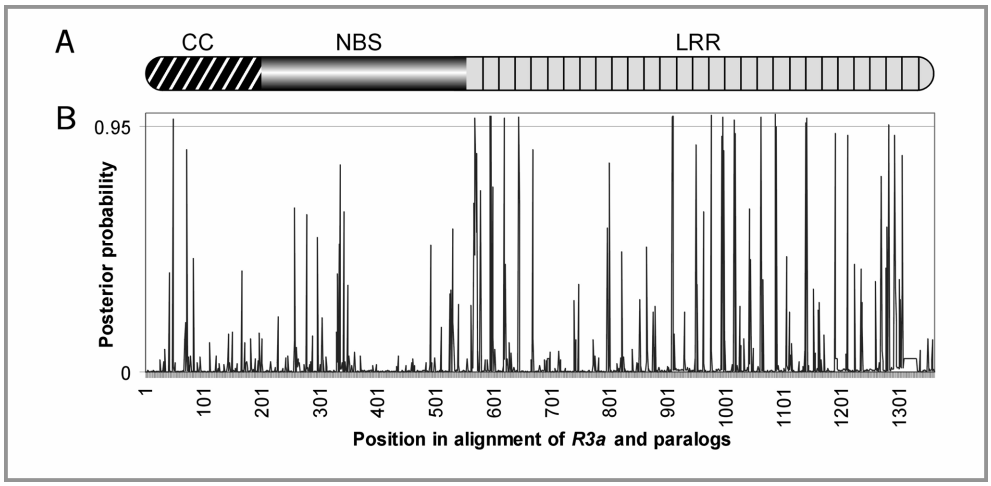


Fig. 5. The distribution of codons under diversifying selection in *R3a*.

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 *I2*GAs 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 *I2*GAs.

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).

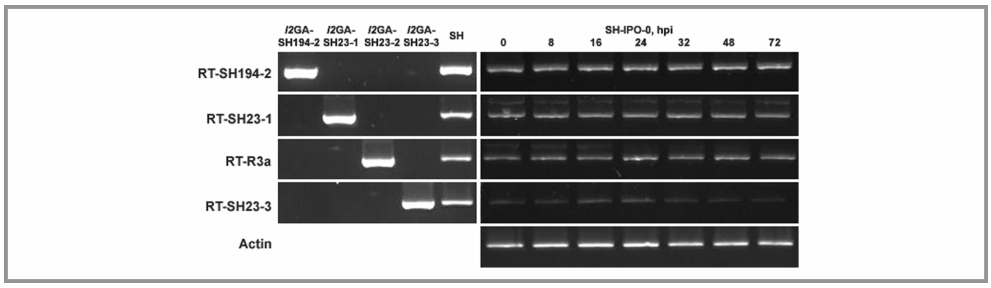


Fig. 7. RT-PCR of the *R3a* cluster. Gene-specific primers used are listed on the left. Subclones with individual *I2*GAs (*I2GA-SH194-2*, *-SH23-1*, *-2*, *-3*) and genomic DNA of SH are used as control for the specificities of the primers. mRNA isolated from the detached leaves of SH inoculated with *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_{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 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

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

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

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

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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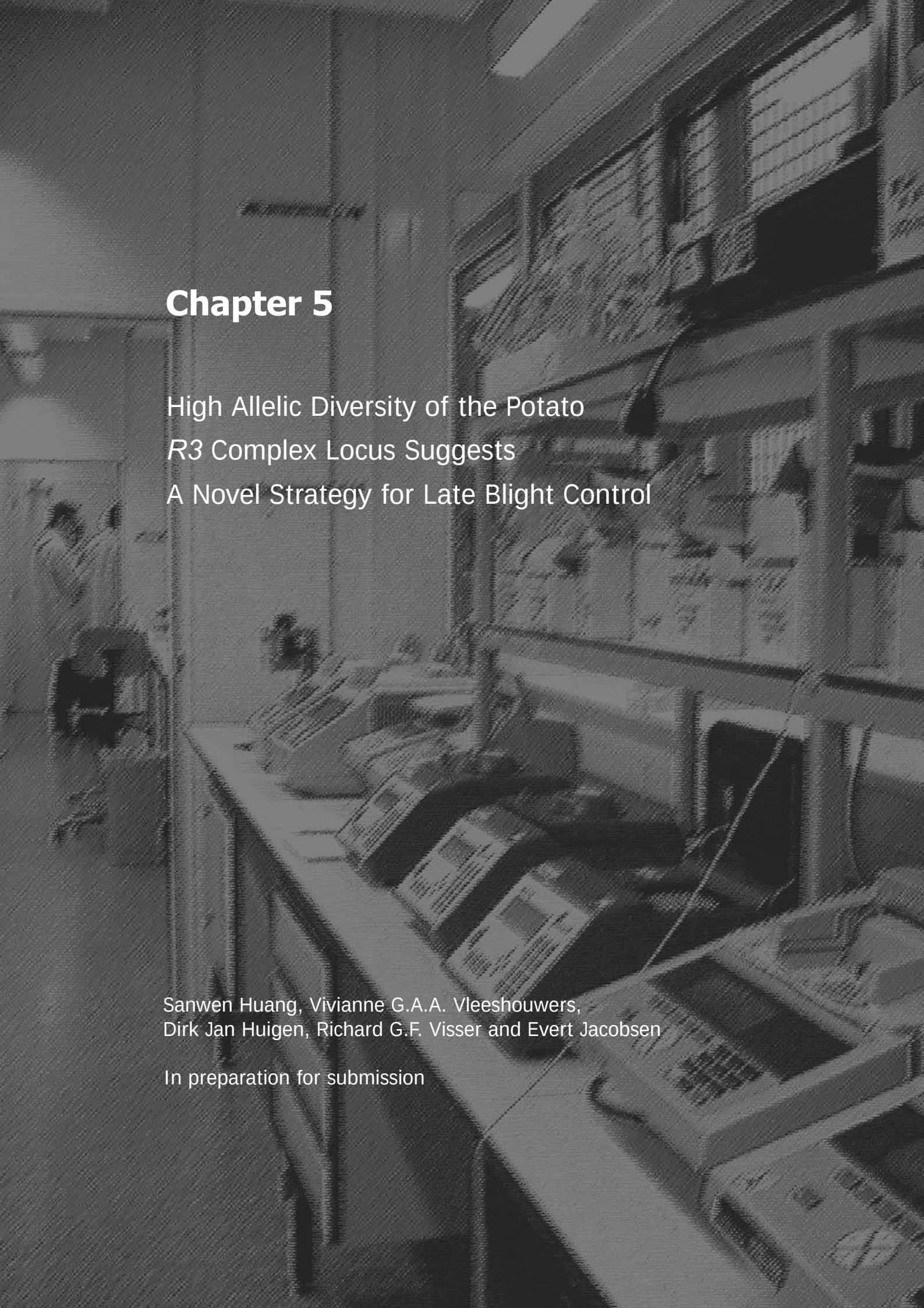

Chapter 5

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*

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 (Müller 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*

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

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

Genotype	Spec. [b]	*R* gene	Pedigree	Remark
RH89-039-16	*tbr*	-	-	*R3* mapping male parent
SH83-92-488	*tbr*	SH *R3* (*R3a, 3b*)	dms 29	*R3* mapping female parent
SW8540-025	*tbr*	*R3a*	SH83-92-488	*R3a* recombinant
SW8540-325	*tbr*	*R3b*	SH83-92-488	*R3b* recombinant
Money Maker	*lyc*	-	-	
Ma [a] *R0*	*tbr*	-	-	Syn. to Bintje
Sc [a] *R0*	*tbr*	-	-	Syn. to Craig Royal
1024-2	*tbr*	-	-	
87-1029-31	*tbr*	-	-	
Desiree	*tbr*	-	-	
Katahdin	*tbr*	-	-	
Pimpernel	*tbr*	-	-	
Robijn	*tbr*	-	-	
Saskia	*tbr*	-	-	
Sirtema	*tbr*	-	-	
BLB256-3	*blb*	*Rpi-blb3*	-	
BLB257-11	*blb*	*Rpi-blb3*	-	
CGN17693	*blb*	R_{ABPT}	-	
CGN21306	*blb*	R_{ABPT}	-	
Ma *R1*	*tbr*	*R1*	W-ras	Syn. to Cebeco43154-5
Sc *R1*	*tbr*	*R1*	CPC2127	Syn. to Craig Snowwhite
Ma *R2*	*tbr*	*R2*	dms 54	Syn. to Cebeco44158-4
Sc *R2*	*tbr*	*R2*	CPC2127	Syn. to 1512 c(16)
Ma *R4*	*tbr*	*R4*	dms 29	Syn. to Cebeco4431-5
Sc *R4*	*tbr*	*R4*	CPC2127	Syn. to 1563 c(14)
CPC2127	*dms*	Sc *R3, R7, R8, R1,2,4*	-	
Sc *R3*	*tbr*	Sc *R3*	CPC2127	Syn. to Pentland Ace
Astarte	*tbr*	Sc *R3*	CPC2127	
Baraka	*tbr*	Sc *R3*	CPC2127	
Cardinal	*tbr*	Sc *R3*	CPC2127	
Element	*tbr*	Sc *R3*	CPC2127	
J92-6462-5	*tbr*	Sc *R3*	CPC2127	dihaploid derivative of Cardinal
Mansour	*tbr*	Sc *R3, R1*	CPC2127	
Patrones	*tbr*	Sc *R3*	CPC2127	
CB67017-018	*tbr*	SH *R3, R1,2*	dms 29	
CB71106-005	*tbr*	SH *R3, R1,2*	dms 29	
Corine	*tbr*	SH *R3*	dms 29	
Escort	*tbr*	SH *R3, R10, R1, 2*	dms 29, MPI19268	
J91-6164-21	*tbr*	SH *R3*	dms 29	dihaploid derivative of Escort
J91-6164-60	*tbr*	SH *R3*	dms 29	dihaploid derivative of Escort
Reaal	*tbr*	SH *R3, R1*	dms 29	
Realta	*tbr*	SH *R3, R1*	dms 29	
Remedy	*tbr*	SH *R3, R1*	dms 29	
Ma *R3*	*tbr*	Ma *R3*	dms 49	Syn. to Cebeco4642-1
Ajax	*tbr*	MPI *R3*	MPI19268	
Radosa	*tbr*	MPI *R3*	MPI19268	
Forelle	*tbr*	FS *R3*	Several dms lines	
Frila	*tbr*	FS *R3*	Several dms lines	
Saphir	*tbr*	FS *R3*	Several dms lines	
707TG11-1	*tbr*	FS *R3, R1,*	Several dms lines	
Ma *R6*	*tbr*	*R6, R6-1, R6-2* [c]	unknown	Syn. to XD2-21
J91-6601-57	*tbr*	*R6*	Ma R6	dihaploid derivative of Ma *R6*
Ma *R7*	*tbr*	*R7*	CPC2127	Syn. to 2182 ef(7)
BE4101-126	*tbr*	*R7*	Ma R7	dihaploid derivative of Ma *R7*
Ma *R8*	*tbr*	*R8*	CPC2127	Syn. to 2424 a(5)
Ma *R9*	*tbr*	*R1-3,9*	CPC2127 and others dms lines	Syn. to 2573 (2)
Ma *R5*	*tbr*	*R5*	unknown	Syn. to 3053-18
Ma *R10*	*tbr*	*R10*	MPI19268	Syn. to 3681 ad (1)
Amigo	*tbr*	*R10*	MPI19268	
Arka	*tbr*	*R10*	MPI19268	
Jaerla	*tbr*	*R10*	MPI19268	
Kondor	*tbr*	*R10*	MPI19268	
Premiere	*tbr*	*R10*	MPI19268	
Producent	*tbr*	*R10*	MPI19268	
Prominent	*tbr*	*R10*	MPI19268	
Provita	*tbr*	*R10*	MPI19268	
Sante	*tbr*	*R10*	MPI19268	
Ma *R11*	*tbr*	*R11*	Several dms lines	Syn. to 5008 ab(6)
4205-1	*tbr*	*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 J91-6601-57 contains and was mapped at the *R3* complex locus.

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

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. RH89-039-16 and SH83-92-488 were used as references to define marker alleles (a for absence and b for presence). The location of the St-I2, R3a, and R3b clusters are marked on the right column.

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*.

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.

A	Allelic version	*R0*	*R3a*	*R3b*	SH *R3*	Ma *R3*	Sc *R3*	FS *R3*	MPI *R3*	*R6*	*R7*	*R8*	*R5*	*R10*	*R11*
	Cultivar/Clone	Bintje	SW8540-025	SW8540-325	SH	Ma *R3*	Sc *R3*	Forelle& Saphir	Ajax& Radosa	J91-6601 57	Ma *R7*	Ma *R8*	Ma *R5*	Ma *R10*	Ma *R11*
P. infestans isolate	89148-9	S	R	R	R	R	R	R	R	R	R	R	R	R	R
	IPO-0	S	R	S	R	R	R	R	R	R	R	R	R	R	R
	89148-07	S	R	S	R	R*	R	R	R	R	R	R	R	R*	R*
	H30P04	S	S	R	R	R	S	S	S	S	S	R	R	R*	R
	90206	S	S	S	S	S	R*	R*	S	S	S	R	R	R	R
	89148-27	S	S	S	S	S	S	S*	S	S	S	R	R	R	R
	USA618	S	S	S	S	S	S	S	S	S	S	R	R	R	S
	90128	S	S	S	S	S	S	S	S	S	S	S	R	R	S
	IPO-82001	S	S	S	S	S	S	S	S	S	S	R	S	S*	S
B	Marker pattern	I	II	II	II	II	II	II	II	II	II	II	III	III	III
	R3a -haplotype	-	I	-	I	nd	I	I	I	I	I	I	II	II	III
	R3b -haplotype	-	-	I	I	nd	II	II	II	-	II	II	I	III	III

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,*

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 *R5* belonged to *R3b-haplotype-I*; Sc *R3*, FS *R3*, MPI *R3*, Ma *R7*, and Ma *R8* fitted in *R3b-haplotye-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.

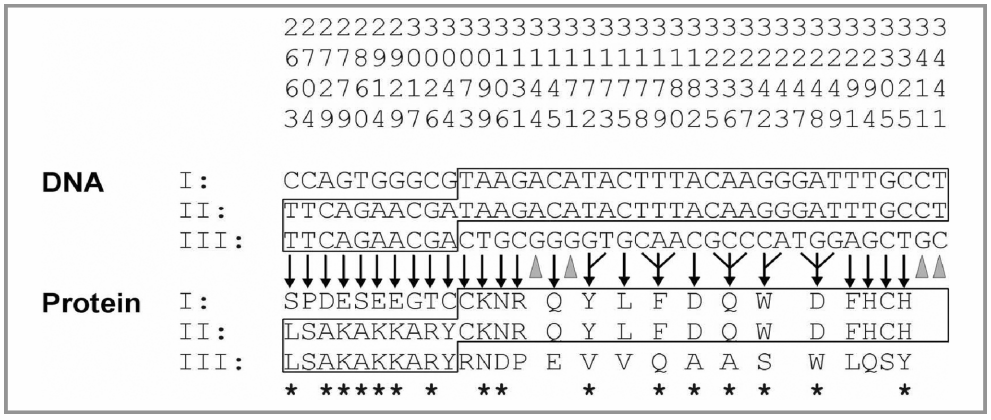

Fig. 1. Allelic variation of the *R3b*-candidate haplotypes (I-III) at both DNA and protein levels. Display of all 38 SNPs. The position of each nucleotide site relative to the first nucleotide of the ATG start codon of the full *R3b*-candidate gene is given by vertical numbers above each site. The DNA 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.

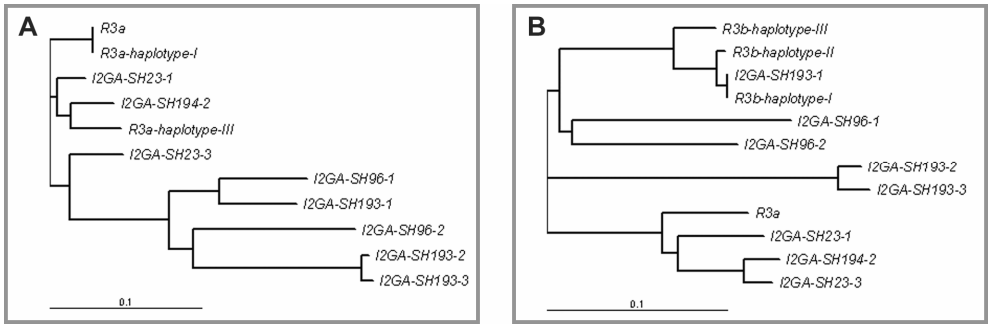

Fig. 2. Distance trees of *R3a*- (A) and *R3b*- (B) haplotypes with *I2*GAs at the *R3a* and *R3b* clusters in SH. Four *I2*GAs (*R3a*, *I2GA-SH23-1*, *-3*, and *I2GA-SH194-2*a) are from the *R3a* cluster and five *I2*GAs (*I2GA-SH96-1*, *-2*, *I2GA-SH193-1* (the *R3b* candidate), *-2*, and -3) are from 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

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.

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

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

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

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-haplotyes* 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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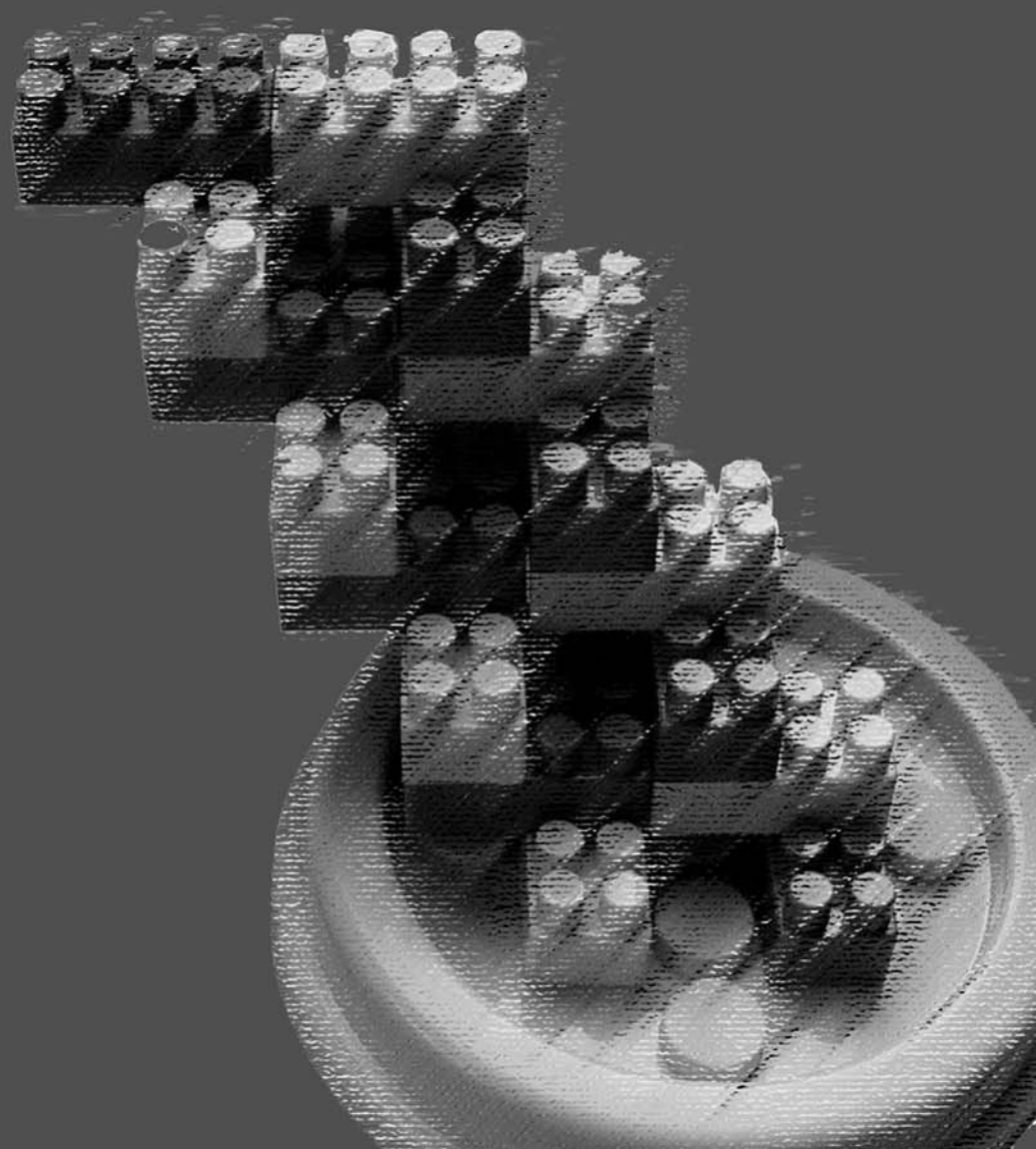
Chapter 6

General Discussion



Sanwen Huang

General discussion

Introduction

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

in vitro Inoculation-a Household Tool

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

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

R Gene Cloning in Potato

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

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

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

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

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

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

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

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

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

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

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

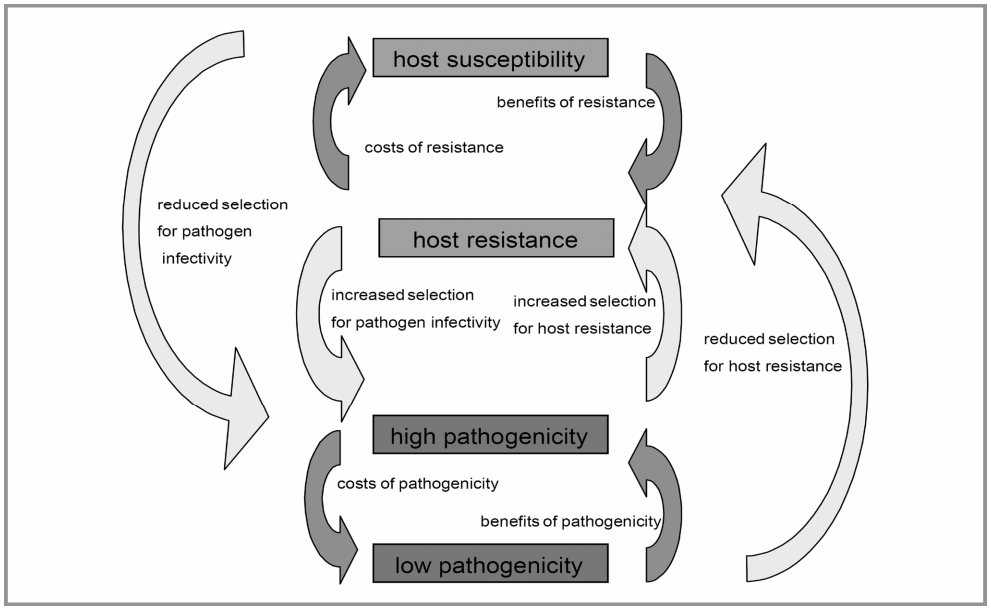


Fig.1 Schematic representation of co-evolution, emphasizing reciprocity in that changes in allele frequencies due to selection in one species impose selection resulting in changes in allele frequencies in the other. Reproduced from Woolhouse et al. 2002.

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

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

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

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

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

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

FUNCTIONAL DIVERSITY OF *R* LOCI

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

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

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

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

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

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

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

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

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

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

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

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Summary

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

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

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

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

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

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

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

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

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

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

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

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

Samenvatting

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

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

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

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

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

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

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

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

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

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

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

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

博士论文

英文题目

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

genomic structure, functional diversity, and implications

中文题目

马铃薯抗晚疫病主效基因位点的发现和分析

基因组结构、功能多态性及对抗病育种的影响

黄三文

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

Curriculum Vitae

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

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

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

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Training and supervision plan of the Graduate School Experimental Plant Sciences

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

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

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

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