Mapping and Cloning of Late Blight Resistance Genes from *Solanum venturii* Using an Interspecific Candidate Gene Approach

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Late blight, caused by the oomycete *Phytophthora infestans*, is one of the most devastating diseases of potato. Resistance (*R*) genes from the wild species *Solanum demissum* have been used by breeders to generate late-blight-resistant cultivars but resistance was soon overcome by the pathogen. A more recent screening of a large number of wild species has led to the identification of novel sources of resistance, many of which are currently being characterized further. Here, we report on the cloning of dominant *Rpi* genes from *S. venturii*. *Rpi-vnt1.1* and *Rpi-vnt1.3* were mapped to chromosome 9 using nucleotide binding site (NBS) profiling. Subsequently, a *Tm-2²*-based allele mining strategy was used to clone both genes. *Rpi-vnt1.1* and *Rpi-vnt1.3* belong to the coiled-coil NBS leucine-rich repeat (LRR) class of plant *R* genes and encode predicted peptides of 891 and 905 amino acids (aa), respectively, which share 75% amino acid identity with the *Tomato mosaic virus* resistance protein *Tm-2²* from tomato. Compared with *Rpi-vnt1.1*, *Rpi-vnt1.3* harbors a 14-aa insertion in the N-terminal region of the protein and two different amino acids in the LRR domain. Despite these differences, *Rpi-vnt1.1* and *Rpi-vnt1.3* genes have the same resistance spectrum.

Late blight, one of the world's most devastating plant diseases, is caused by the oomycete *Phytophthora infestans*, causing an estimated yearly economic loss of \$3.25 billion in potato-growing areas worldwide. Despite more than 150 years of resistance breeding, disease management still relies on the enormous application of fungicides. Breeding for late blight resistance was stimulated in the mid-nineteenth century by the disastrous consequences of *Phytophthora* epidemics in the United States and Europe. Only a few of the many existing landraces survived and these formed the basis for cultivar development. However, until 1910, the reduced aggressiveness of the pathogen since 1850 probably played a more prominent role in the survival of the potato crop in Europe than the first breeding activities. The rediscovery of the Mendelian laws of

genetics subsequently directed the focus of potato resistance research on the use of wild *Solanum* spp. grown in South and Central America. Initially, special attention was given to *Solanum demissum* as the main resistance source (Black and Gallegly 1957; Malcolmson and Black 1966). To date, at least 11 specificities (*R1–R11*) have been identified in *S. demissum*, four of which (*R1*, *R2*, *R3*, and *R10*) have been introgressed by breeders up to cultivar level; however, races of the pathogen that were able to overcome these genes emerged within a few years after market introduction (Turkensteen 1993). By the end of the 1950s, most breeders switched to the use of sources of germplasm with partial or quantitative resistance, the underlying paradigm being that this type of resistance is resistant (*R*)-gene independent and assumed to be of polygenic nature and, thus, more durable (Turkensteen 1993). Extensive research in this area has led to the identification of many minor quantitative trait loci (QTL) (Bradshaw et al. 2006b; Collins et al. 1999; Costanzo et al. 2005); however, stacking of these QTL has proven to be very difficult, due to the outbreeding nature of cultivated potato. Moreover, this type of resistance is strongly correlated with late maturity under long day conditions (Howard et al. 1970). This, together with the finding that quantitative resistance is apparently also amenable to erosion (Flier et al. 2003), has stimulated breeders to reconsider the use of *R* genes, preferably through stacking of complementary genes.

A recent comprehensive survey of wild tuber-bearing *Solanum* spp. revealed that the genus *Solanum* harbors a wealth of late-blight-resistance sources that have yet to be exploited. Recent studies into the genetic basis of late-blight resistance in *S. demissum* showed that 8 of the 11 known specificities—*R3* (now known to be *R3a* and *R3b*), *R5*, *R6*, *R7*, *R8*, *R9*, *R10*, and *R11*—are located close to each other on chromosome 11 (Bradshaw et al. 2006a; El Kharbotly et al. 1994; Huang et al. 2004, 2005). Other mapped *R* genes from *S. demissum* include *R2* on chromosome 4 (Li et al. 1998) and *R1* on chromosome 5 (El Kharbotly et al. 1994; Leonards-Schippers et al. 1992). In addition to *S. demissum*, other wild *Solanum* spp. have been recognized as sources of late blight resistance. The described *R* loci include *Rpi-ber1* (initially named *R_{ber}*) from *S. berthaultii* on chromosome 10 (Ewing et al. 2000); *RB/Rpi-blb1*, *Rpi-blb2*, and *Rpi-blb3* from *S. bulbocastanum* on chromosome 8, 6, and 4, respectively (Naess et al. 2000; Park et al. 2005; van der Vossen et al. 2003, 2005); *Rpi-pnt1* (initially named *Rpi1*) (Kuhl et al. 2001) from *S. pinnatisectum* on chromosome 7; *Rpi-mcq1* (initially named *Rpi-moc1*) (Smilde et al. 2005)

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from *S. mochiquense* on chromosome 9; and *Rpi-phu1* from *S. phureja* on chromosome 9 (Sliwka et al. 2006). In addition, several QTL involved in resistance to late blight have been reported, in both cultivated potato (Gebhardt and Valkonen 2001) and wild species, such as *S. microdontum* (Sandbrink et al. 2000; Tan et al. 2008), *S. paucissectum* (Villamon and Spooner 2005), and *S. phureja* on chromosome 7 and 12 (Ghislain et al. 2001).

In contrast to introgression breeding, isolation of *R* genes from *Solanum* spp. and their stable transformation into existing potato cultivars is by far the fastest means of exploiting potentially durable late-blight resistance present in the *Solanum* gene pool. Cloning of *R* genes is typically done through a positional cloning strategy. Currently, four *R* genes for late-blight resistance have been cloned and all belong to the coiled-coil nucleotide-binding site leucine-rich repeat (CC-NBS-LRR) class of plant *R* genes: *R1* (chromosome 5) and *R3a* (chromosome 11) from *S. demissum* (Ballvora et al. 2002; Huang et al. 2005) and *Rpi-blb1* and *Rpi-blb2* from *S. bulbocastanum* on chromosomes 8 and 4, respectively (Naess et al. 2000; van der Vossen et al. 2003, 2005). Once a functional gene is cloned from a specific *R* locus, one can try to clone functional alleles from the same or different species in order to determine allele frequency and allelic variation at a given locus. This was illustrated by the cloning of *Rpi-sto1* and *Rpi-ptal* from *S. stoloniferum* and *S. papita*, respectively, which were shown to be functional alleles of *Rpi-blb1* (Vleeshouwers et al. 2008). Here, we demonstrate that NBS profiling (van der Linden et al. 2004), when combined with bulked segregant analysis (BSA) (Michelmore et al. 1991), is a powerful tool to generate candidate gene markers. They can predict the position of the *R* locus under study and, upon sequence information, form a starting point for cloning of the gene through a functional allele mining strategy, avoiding the map-based cloning approach. Depending on the resolution of relevant genetic mapping studies and the size of the candidate gene family, an allele mining approach can generate many candidate genes which need to be functionally analyzed.

To date, functional analysis of candidate *R* gene homologs (RGH) typically requires stable transformation of a susceptible genotype for complementation purposes. This is a time-consuming and inefficient approach because it takes at least several months to generate transgenic plants that can functionally be analyzed. In the current study, we have exploited the finding that *Nicotiana benthamiana* is susceptible to *P. infestans* (Becktell et al. 2006) to develop a transient complementation assay for *R* genes that confer resistance against *P. infestans* (H. Rietman, I. Hein, R. G. F., Visser, E. A. G. Van der Vossen, and V. G. A. A. Vleeshouwers, *unpublished results*).

Here, we present an example of how we used the above-described approaches or techniques to clone two functionally equivalent *R* genes from *S. venturii*, a species with its origin in the Andean region of South America.

RESULTS

Genetic basis and spectrum of late blight resistance in *S. venturii*.

To determine the genetic basis of late blight resistance in *S. venturii*, 14 *S. venturii* accessions were screened in detached-leaf assays (DLA) with the *P. infestans* isolate IPO-C. Resistant genotypes selected from the *S. venturii* accessions CGN18108 and CGN18000 were used to generate the mapping populations 7698 and 7663, respectively. Following DLA with 52 F1 progeny plants of population 7698, 30 were scored as resistant and 22 as susceptible, suggesting the presence of a single dominant *R* gene (*Rpi-vnt1.1*). Of the 60 F1 progeny plants screened from population 7663, 24 were scored as resistant and 36 as susceptible, suggesting that *vnt365-1* also contained a single dominant *R* gene (*Rp-vnt1.3*). Resistance genes were named in agreement with the concurrent work of Foster and associates (2009) who identified in the *S. venturii* accessions CGN18108, CGN18279, and CGN18000 three single dominant *R* genes, *Rpi-vnt1.1*, *Rpi-vnt1.2*, and *Rpi-vnt1.3*, respectively.

The resistance spectra of *Rpi-vnt1.1* and *Rpi-vnt1.3* were analyzed by challenging them with several isolates of different complexity and aggressiveness (Table 1). Both genes were only overcome by strain EC1, suggesting that they share the same resistance spectrum.

Mapping of *Rpi-vnt1.1* and *Rpi-vnt1.3* to chromosome 9.

In an attempt to develop markers linked to *Rpi-vnt1.1* and *Rpi-vnt1.3*, we carried out a BSA in combination with NBS profiling in both mapping populations. This led to the identification of nine bulk-specific markers for *Rpi-vnt1.1* in 7698 and eight for *Rpi-vnt1.3* in 7663. On the full offspring, only two resistant bulk-specific fragments, one generated with the NBS2/*RsaI* primer-enzyme combination and the other with NBS3/*HaeIII*, cosegregated with resistance in both initial 7698 and 7663 populations of 52 and 60 F1 progeny plants, respectively. Therefore, these fragments were cloned and sequenced. When subjected to a BLAST analysis, both sequences turned out to be highly similar to the *Tm-2²* gene on chromosome 9 of tomato (Lanfermeijer et al. 2003). The cloned NBS2/*RsaI* and NBS3/*HaeIII* fragments were 350 and 115 bp in size and shared 88.3 and 80.3% DNA sequence identity, respectively, with *Tm-2²*, suggesting that *Rpi-vnt1.1* and *Rpi-vnt1.3* were *Tm-2²* related and, thus, could lie on chromosome 9. Genetic linkage of the NBS fragments to the *R* genes under study was verified by developing fragment-specific SCAR (sequence-characterized amplified region) markers in the *Rpi-vnt1.1* and *Rpi-vnt1.3* mapping populations. In this way, the NBS3-derived marker NBS3b was mapped relative to the chromosome-9-derived markers TG35, TG551, TG186, CT183, or T1421 (Fig. 1). Linkage of TG35 and TG186, and TG35 and TG551, to *Rpi-vnt1.1* and *Rpi-vnt1.3*, respectively, indicated that both genes were indeed derived from similar regions on chromosome 9 (Fig. 1). These findings were in line with the result of

Table 1. Description of *Phytophthora infestans* isolates used to determine the specificity of *Rpi-vnt1.1* and *Rpi-vnt1.3*

Isolate ID	Country of origin	Isolation year	Race	<i>Rpi-vnt1.1</i> and <i>Rpi-vnt1.3</i> phenotypes
90128	Geldrop, The Netherlands	1990	1.3.4.7.(8)	Resistant
H30P04	The Netherlands	Unknown	7	Resistant
IPO-C	Belgium	1982	1.2.3.4.6.7.10.11	Resistant
USA618	Toluca Valley, Mexico	Unknown	1.2.3.6.7.11	Resistant
VK98014	Veenkolonien, The Netherlands	1998	1.2.4.11	Resistant
IPO-428-2	The Netherlands	1992	1.3.4.7.8.10.11	Resistant
NL00228	The Netherlands	2000	1.2.4	Resistant
Katshaar	Katshaar, The Netherlands	Unknown	1.3.4.7.10.11	Resistant
F95573	Flevoland, The Netherlands	1995	1.3.4.7.10.11	Resistant
89148-09	The Netherlands	1989	0	Resistant
EC1	Ecuador	Unknown	3.4.7.11	Susceptible

concurrent work of Foster and associates (2009), who followed a map-based cloning approach to clone *Rpi-vnt1.1*, *Rpi-vnt1.2*, and *Rpi-vnt1.3* genes.

In order to develop flanking markers to screen a large offspring for recombinant events, markers linked to TG35 or TG551 were selected from Solanaceae Genomics Network (SGN) and screened in both populations. Despite low levels of polymorphism, expressed sequence tag-based markers U276927 and U270442 have been developed and mapped in populations 7698 and 7663, respectively (Table 2; Fig. 1). U276927 could be mapped 1.8 centimorgans (cM) north of *Rpi-vnt1.1* whereas

U270442 was mapped 3.5 cM south of *Rpi-vnt1.3*. Subsequently, recombinant offspring was identified using 500 individuals of population 7698 and 1,005 individuals of population 7663, using the flanking markers U276927/TG186 and NBS3B/U270442, respectively. This resulted in the mapping of *Rpi-vnt1.1* and *Rpi-vnt1.3* within a genetic intervals of 4 and 3.7 cM, respectively (Fig. 1).

Tm-2-based allele mining.

In view of the expected high DNA sequence homology between *Rpi-vnt1.1*, *Rpi-vnt1.3*, and *Tm-2*², we adopted a homology-based allele mining strategy to clone the former two genes. The first step was to design degenerated primers incorporating the putative start and stop codons of candidate *Tm-2*² gene homologs (*Tm2GH*). Based on an alignment of all the available potato- and tomato-derived *Tm-2*²-like sequences in public sequence databases, we designed primers ATG-Tm2F and TGA-Tm2R (Table 3). However, no amplicons of the expected size were generated when this primer set was tested on the parental genotypes of both mapping populations. Because the ATG-Tm2F primer sequence was present in the cosegregating NBS profiling-derived marker sequences, three new reverse primers (REV-A, REV-B, and REV-C) were designed 100 bp upstream of the initial TGA-Tm2R primer site, in a region that was conserved in all the aligned *Tm-2*²-like sequences. When combined with either ATG-Tm2F or NBS3BF, a single amplicon of approximately 2.5 kb was specifically amplified from the resistant parental genotypes only (i.e., vnt7014-9 and vnt365-1). These fragments were cloned into the pGEM-T Easy vector and approximately 96 individual clones from each genotype were sequenced using a primer walk strategy. All the obtained sequences shared 75 to 80% similarity to *Tm-2*². A total of five different classes could be distinguished within the vnt7014-9-derived sequences whereas the vnt365-1-derived sequences fell into only three different classes. These different classes were subsequently named NBS3B-like or non-NBS3B-like based on the degree of homology to the NBS3B sequence.

Fig. 1. Genetic linkage maps of chromosome 9 of the **a**, *Rpi-vnt1.1* and **b**, *Rpi-vnt1.3* loci mapped in the populations 7698 and 7663, respectively. Numbers on the left side indicate genetic distances (centimorgans). Relative positions of mapped loci are indicated by horizontal lines. The letter n represents the size of each population.

Table 2. Markers used to map *Rpi-vnt1.1* and *Rpi-vnt1.3*

Marker	Primer orientation	Primer sequence	Annealing temperature (°C)	Enzyme ^a
NBS3B	Forward	ccttctcctcctcacatttag	65	a.s.
	Reverse	gcattgccaactattgaacaac	...	...
TG35	Forward	cacggagactaagattcagg	60	<i>HhaI/XapI</i> (c)
	Reverse	taaaggtgatgctgatgggg	...	...
TG551	Forward	ccagaccaccaagtgtgttc	58	<i>TaqI</i> (c)
	Reverse	aactttcagatatgctctgcag	...	...
TG186	Forward	aacggtgtacgagattttac	58	<i>HphI</i> (c)
	Reverse	acctacatagatgaacctcc	...	...
U270442	Forward	ggatattatcttgcaacatctcg	55	<i>XapI</i> (r)
	Reverse	cttctgatggtatgcatgagaac	...	...
U276927	Forward	gcattagcgcaattggaatccc	58	<i>HphI</i> (c)
	Reverse	ggagagcattagtacagcgctc	...	...

^a Abbreviations: a.s. = allele specific, c = coupling phase, and r = repulsing phase.

Table 3. Primers used for various experiments as described in this article

Marker	Primer orientation	Primer sequence	Annealing temperature (°C)	Experiments
NBS-GSP1	Forward	tccaaatattgtcgagttggg	Touch down	Genome walking
NBS-GSP2	Forward	gctttggtgcagacatgatgc	Touch down	Genome walking
REV-A	Reverse	ggtgtcgaagtaacgtgcac	55	<i>Tm2</i> -based allele mining
REV-B	Reverse	tgcacggatgatgtcagatgcc	55	<i>Tm2</i> -based allele mining
REV-C	Reverse	caacttgaagtttgcatttc	55	<i>Tm2</i> -based allele mining
ATG-Tm2F	Forward	atggctgaaattctctcacagc	55	<i>Tm2</i> -based allele mining
TAA-8bisR	Reverse	ttatagtacctgtatattctcaac	55	<i>Tm2</i> -based allele mining
ATG2-Tm2F	Forward	atgaattattgtttacaagacttg	55	<i>Tm2</i> -based allele mining
TGA-Tm2R	Reverse	tgatattctcaacttgaagc	55	<i>Tm2</i> -based allele mining
GSP1-5race	Reverse	gaacactcaaattgatgacagacatgcc	67	5' rapid amplification of cDNA ends
GSP2-5race	Reverse	cccaaaccggcgcatccaactattg	67	5' rapid amplification of cDNA ends

In order to retrieve the missing C-terminal part of the amplified *Tm2GH*, a 3'-genome walk was performed using primers NBS-GSP1 and NBS-GSP2 (Tables 2 and 3), which were designed approximately 100 bp upstream of the REV-A, REV-B, and REV-C primers, in order to generate an overlap of 100 bp between the cloned NBS3B-like sequences and clones generated with the genome walk. Three amplicons of approximately 200 bp were obtained from vnt7014-9 and a single clone of approximately 1 kb from vnt365-1. Following cloning, sequencing, and alignment to the cloned *Tm2GH*, all four clones seemed to fit to clone *Tm2GH*-vnt8b, because the overlapping 100 bp were an exact match. To be able to subsequently amplify full-length *Tm2GH* from the *Rpi-vnt1.1* and *Rpi-vnt1.3* loci, we designed a novel reverse primer (TAA-8bR) (Tables 2 and 3) based on the alignment of the full-length *Tm2GH*-vnt8b sequence with the *Tm-2²* sequence from tomato (Fig. 2). The original TGA stop codon was not present in the *Tm2GH*-vnt8b sequence; therefore, we included the next in-frame stop-codon (TAA) which was situated 12 bp downstream.

Full-length amplification of *Tm2GH* from vnt7014-9 and vnt365-1 was subsequently pursued with high-fidelity *Pfu* Turbo polymerase using primers ATG-Tm2F and TAA-8bR. Amplicons of approximately 2.6 kb were cloned into the pGEM-T Easy vector and sequenced. Three different types of clones were obtained from vnt7014-9, one of which harbored an open reading frame (ORF) of the expected size (*Tm2GH*-vnt1b). All the clones obtained from vnt365-1 were identical to each other and contained the expected ORF. Clone *Tm2GH*-vnt1.9 was chosen together with *Tm2GH*-vnt1b for further genetic analysis.

Before targeting *Tm2GH*-vnt1b and *Tm2GH*-vnt1.9 for complementation analysis, we needed to confirm that the selected *Tm2GH* indeed mapped to the *Rpi-vnt1.1* and *Rpi-vnt1.3* loci. When tested as SCAR markers in the initial mapping populations, both markers cosegregated with resistance. Upon amplification with ATG-Tm2F and TAA-8bisR in the set of recombinants which defined the *Rpi-vnt1.1* and *Rpi-vnt1.3* loci, amplicons of the expected size were indeed only gener-

ated from late-blight-resistant recombinants, confirming that both *Tm2GH* were indeed good candidates for *Rpi-vnt1.1* and *Rpi-vnt1.3*. However, there were resistant recombinants, two in the *Rpi-vnt1.1* mapping population and one in the *Rpi-vnt1.3* mapping population, which did not give the expected polymerase chain reaction (PCR) product, suggesting that both loci could in fact harbor a tandem of two functional *R* genes.

Transient complementation using *Agrobacterium* transient transformation assays in *N. benthamiana*.

To investigate whether *Tm2GH*-vnt1b and *Tm2GH*-vnt1.9 were functional *R* genes, we inserted them into a Gateway binary expression vector in between the regulatory elements of the *Rpi-blb3* gene (Lokossou et al. in press). *N. benthamiana* leaves were then infiltrated with *Agrobacterium tumefaciens* cultures containing the relevant clones. Two days postinfiltration, the leaves were challenged with *P. infestans* in a DLA. Two different isolates were used, EC1 and IPO-C, which show a differential response to *Rpi-vnt1.1* and *Rpi-vnt1.3*. Both genes confer resistance to IPO-C but allow for a compatible interaction in the case of EC1. Three independent transient complementation assays were carried out in triplicate with both isolates. For each replicate, leaf numbers 4, 5, and 6 (when counting from the bottom of the plant) were agroinfiltrated and subsequently challenged with *P. infestans*. At 6 days postinoculation with IPO-C, leaves transiently expressing *Tm2GH*-vnt1b or *Tm2GH*-vnt1.9 displayed an infection efficiency between 40 and 60% (Fig. 3A and B). The resistant control plants transiently expressing the functional *Rpi-sto1* gene (Vleeshouwers et al. 2008) showed a significantly lower infection efficiency ranging between 10 and 20%, (80 to 90% of the challenged leaves showed a hypersensitive response [HR]). In contrast, leaves expressing abptGH-a, a nonfunctional paralog of *Rpi-abpt* (Lokossou et al. in press) were fully susceptible (Fig. 3A and B). In the case of EC1, all agroinfiltrated leaves were susceptible except for those infiltrated with *Rpi-sto1*, which confers resistance to EC1 (Fig. 3A and C). These

Fig. 2. Alignment of the *Tm-2²* open reading frame with two polymerase chain reaction fragments obtained with a Genome Walking kit (GW1 and GW2). The stop codons of the *Tm-2²* gene and *Rpi-vnt1.1* / *vnt1.3* are presented in blue and red, respectively.

data matched with the resistance spectrum of *Rpi-vnt1.1* and *Rpi-vnt1.3*, suggesting that *Tm2GH-vnt1b* and *Tm2GH-vnt1.9* indeed represented *Rpi-vnt1.1* and *Rpi-vnt1.3*, respectively.

Complementation analysis through stable transformation of cv. Desiree.

To confirm the results obtained with the transient complementation assays in *N. benthamiana*, the binary Gateway constructs

harboring *Tm2GH-vnt1b* and *Tm2GH-vnt1.9* were transferred to the susceptible potato cv. Desiree through *Agrobacterium*-mediated transformation. As a resistant control, we also transformed cv. Desiree with construct pSLJ21152, a binary construct harboring a 4.3-kb fragment carrying the putative *Rpi-vnt1.1* promoter, ORF, and terminator sequence (Foster et al. 2009). Primary transformants harboring the transgenes of interest were tested for resistance to *P. infestans* in DLA. Surpris-

Fig. 3. Transient complementation assays in *Nicotiana benthamiana*. **A**, Typical detached leaf assay responses of *N. benthamiana* leaves infiltrated with either *Tm2GH-vnt1b*, *Tm2GH-vnt1.9*, *Rpi-sto1* (resistant control), or *abptGH-a* (susceptible control). The top row shows the response to IPO-complex (non-virulent isolate) whereas the bottom row shows the response to EC1 (virulent isolate). *Rpi-sto1* gives resistance to both isolates as expected. Pictures were taken 6 days postinoculation. **B** and **C**, Quantification of infection efficiency in the transient complementation assay as illustrated in **A**. *Tm2GH-vnt1b* and *Tm2GH-vnt1.9* showed a higher infection efficiency, ranging from 40 to 60%, than the resistant control *Rpi-sto1* construct during an incompatible interaction with IPO complex isolate.

ingly, only the genetic construct harboring the 4.3-kb *Rpi-vnt1.1* fragment was able to complement the susceptible phenotype; eight of nine primary transformants were resistant. All 22 *Tm2GH-vnt1b* and 17 *Tm2GH-vnt1.9* containing primary transformants were susceptible to *P. infestans*.

Alignment of the *Tm2GH-vnt1b* and *Tm2GH-vnt1.9* sequences to the 4.3-kb *Rpi-vnt1.1* fragment revealed the presence of an additional ATG start codon 99 nucleotides (nt) upstream from the start codon that was used as basis for the PCR-based allele-mining experiments exploiting *Tm-2²* homology. This finding, together with the negative complementation results obtained with the *Tm2GH-vnt1b* and *Tm2GH-vnt1.9* and the positive complementation result with 4.3-kb *Rpi-vnt1.1* fragment, suggest that the 5' most upstream start codon represents the actual start of the functional *Rpi-vnt1.1* and *Rpi-vnt1.3* genes.

Transient complementation assays using extended allele mining products.

In an attempt to PCR amplify the putatively full-length *Rpi-vnt1.1* and *Rpi-vnt1.3* genes from vnt7014-9 vnt365-1, respectively, genomic DNA of both genotypes was subjected to long-range PCR using the primers ATG2-Tm2F and TAA-8bR (Table 3). Amplicons of the expected size were cloned into the pGEM-T Easy vector and sequenced. Clones obtained from vnt7014-9 were all the same and identical to the corresponding sequence in pSLJ21152 (Foster et al. 2009). Clones obtained from vnt365-1 were also all identical but contained an insertion of 42 nt in the 5' extended region compared with those obtained from vnt7014-9. Both sequences were subsequently inserted into the Gateway binary expression vector in between the regulatory elements of the *Rpi-blb3* gene (Lokossou et al. in press) and targeted for transient complementation analysis in *N. benthamiana*, together with the original *Tm2GH-vnt1b* and *Tm2GH-vnt1.9* constructs and pSLJ21152. Both full-length genes showed infection efficiency comparable with the 4.3-kb genomic clone harboring the *Rpi-vnt1.1* gene (10 to 30% of the challenged leaves showed typical symptoms of late blight), whereas the shorter gene constructs again displayed significantly higher infection efficiency (65 to 75% of infected leaves), indicating that the full-length amplicons derived from vnt7014-9 and vnt365-1 represent *Rpi-vnt1.1* and *Rpi-vnt1.3*, respectively (Fig. 4A, B, and C).

Complementation analysis through stable transformation of cv. Desiree with the full-length PCR products.

To confirm the results obtained with the second transient complementation assays in *N. benthamiana*, the binary Gateway constructs harboring the full-length candidate genes *Tm2GH-vnt1.1FL* and *Tm2GH-vnt1.3FL* were transferred to the susceptible potato cv. Desiree through *Agrobacterium*-mediated transformation. Primary transformants harboring the transgenes of interest were tested for resistance to *P. infestans* in DLA. As expected, both construct were able to complement the susceptible phenotype (Fig. 5). In all, 13 of 15 and 14 of 20 primary transformants with *Tm2GH-vnt1.1FL* and *Tm2GH-vnt1.3FL*, respectively, were resistant. This result confirmed the results observed in the transient assay. Therefore, the cloned PCR products *Tm2GH-vnt1.1FL* and *Tm2GH-vnt1.3FL* were demonstrated to be *Rpi-vnt1.1* and *Rpi-vnt1.3*, respectively.

Gene structure of *Rpi-vnt1.1* and *Rpi-vnt1.3*.

The 5'-terminal structure of *Rpi-vnt1.1* and *Rpi-vnt1.3* was determined by comparing the genomic sequences with cDNA fragments generated by 5' rapid amplification of cDNA ends (RACE). For *Rpi-vnt1.1* and *Rpi-vnt1.3*, RACE identified 5' cDNA fragments comprising 5' untranslated regions of 83 and

43 nt, respectively. The ORF of *Rpi-vnt1.1* and *Rpi-vnt1.3* encode predicted peptides of 891 and 905 amino acids, respectively. In addition to the 14-amino-acid insertion in the N-terminal region of *Rpi-vnt1.3*, only two other amino acids differ between *Rpi-vnt1.1* and *Rpi-vnt1.3*. At positions 548 and 753, *Rpi-vnt1.1* harbors an asparagine and arginine residue whereas the corresponding residues in *Rpi-vnt1.3* are tyrosine and lysine, respectively (Fig. 6). However, the substituted residues have the same characteristics. Asparagine and tyrosine belong to the group of hydrophobic residues whereas arginine and lysine are positively charged residues. The protein sequences of both genes harbor several conserved motifs of the CC-NBS-LRR class of R proteins (Fig. 6). A CC domain is located in the N-terminal parts of the proteins between amino acids 1 and 183 for *Rpi-vnt1.1* and between 1 and 198 for *Rpi-vnt1.3*. In the first 183 or 198 residues, two pairs of putative heptad motifs composed of hydrophobic residues could be recognized in *Rpi-vnt1.1* and *Rpi-vnt1.3* sequences, respectively. An NBS-ARC (apoptosis, R gene products, CED-4) domain could be recognized in the amino acid stretch between residues 183 or 198 and 472 or 486, respectively (P-loop, Kinase-2, GLPL) (Van der Biezen and Jones 1998). The C terminal part of *Rpi-vnt1.1* and *Rpi-vnt1.3* comprises a series of 15 LRR motifs of irregular size that can be aligned according to the consensus sequence LxxLxxLxxLxxLxxC/N/Sx(x)LxxLPxx (where x is any amino acid) (McHale et al. 2006). A PROSITE analysis (Hofmann et al. 1999) identified 4 N-glycosylation sites, 7 casein kinase II phosphorylation sites, 10 protein kinase C phosphorylation sites, 6 N-myristoylation sites, and 1 cAMP- and cGMP-dependent protein kinase phosphorylation site.

At the protein level, *Rpi-vnt1.1* and *Rpi-vnt1.3* share 73% amino acid sequence identity with *Tm-2²*. Interestingly, the lowest percentage of similarity was found in the LRR domain, where *Rpi-vnt1.1* and *Rpi-vnt1.3* share 62% amino acid sequence identity with *Tm-2²*. In contrast, the CC and NBS-ARC domains of *Rpi-vnt1.1* and *Rpi-vnt1.3* share 86.5% amino acid sequence identity with the corresponding regions of *Tm-2²*.

Mapping of *Tm2GH* in the ultradense genetic map of potato.

As previously described, two groups of *Tm2GH* were found based on the degree of homology to the NBS3B sequence: NBS3B-like and non-NBS3B-like. The *Rpi-vnt1.1* and *Rpi-vnt1.3* candidate genes *Tm2GH-vnt1b* and *Tm2GH-vnt1.9*, respectively, were from the NBS3B-like group and segregated with resistance. In an attempt to determine the chromosomal positions of other *Tm2GH* belonging to the non-NBS3B-like sequences group, we used the primers ATG-Tm2-F combined with REV-C to develop *Tm2GH*-specific markers in the diploid reference mapping population, a cross between the diploid potato genotypes SH83-92-488 (SH) and RH89-039-16 (RH) (SH × RH), which was previously used to generate the ultradense genetic map of potato (Isidore et al. 2003; van Os et al. 2006). Following digestion of the PCR amplicons derived from SH, RH, and five F1 progeny plants with different restriction enzymes, we identified four enzymes to generate CAPS markers that segregated from RH (*Cfr131*, *Hin1II*, *AluI*, and *HhaI*) and one that segregated from SH (*HpyF10IV*). Subsequent mapping of these markers in the complete SH × RH mapping population positioned them at the bottom of chromosome 9, a region of the chromosome that is similar to the late-blight R gene *Rpi-mcql* (Fig. 7A and B) (Lanfermeijer et al. 2003; Sliwka et al. 2006; Smilde et al. 2005; Trognitz and Trognitz 2005). The mapped fragments shared between 85 and 90% homology (DNA level), with *Rpi-vnt1.1*, *Rpi-vnt1.3*, and *Tm-2²* revealing the presence of a third *Tm-2²*-like cluster at

Fig. 4. Functional comparison of the N-terminal part of the truncated and full-length *Rpi-vnt1.1* and *Rpi-vnt1.3* gene constructs. **A**, Transient complementation assays in *Nicotiana benthamiana* were repeated to compare infection efficiency of the full-length candidate genes *Tm2GH-vnt1.1FL* and *Tm2GH-vnt1.3FL* with the truncated ones, *Tm2GH-vnt1b* and *Tm2GH-vnt1.9*. The construct pSLJ21152, harboring a 4.3-kb fragment carrying the putative *Rpi-vnt1.1* promoter, open reading frame (ORF), and terminator sequence was used as resistant control. The nonfunctional resistance gene *abpt-RGH-a* was chosen as susceptible control. Candidate genes *Tm2GH-vnt1.1FL* and *Tm2GH-vnt1.3FL* show an infection efficiency as low as the resistant control (construct pSLJ21152) whereas the truncated candidate genes show a higher infection efficiency. **B** and **C**, Quantification of infection efficiency in the transient complementation assay as illustrated in **A**. *Tm2GH-vnt1.1FL* (full length of *Rpi-vnt1.1* candidate gene) and *Tm2GH-vnt1.3FL* (full length of *Rpi-vnt1.3* candidate gene) showed a level of infection similar to the resistant control, pSLJ21152 (4.3-kb fragment carrying the putative *Rpi-vnt1.1* promoter, ORF, and terminator sequence).

the distal end of the long arm of the chromosome 9. Alignment of mapped fragments shows that they all cluster with *Tm2GHs* in a different group than *Rpi-vnt1.1* and *Rpi-vnt1.3*. Therefore, *Rpi-mcq1* is likely to be a *Tm2GH* as well.

DISCUSSION

Here, we report on the cloning of two putatively allelic late-blight *R* genes, *Rpi-vnt1.1* and *Rpi-vnt1.3*, from the wild potato species *S. venturii*, using a candidate gene allele-mining approach. The candidate gene family was identified through NBS profiling (van der Linden et al. 2004) in combination with a BSA approach. Sequences of NBS marker bands genetically linked to the resistance phenotypes suggested that the target genes were located in an area of the potato genome that harbored *R* gene homologs that were highly homologous to the *Tomato mosaic virus* resistance (ToMV) gene *Tm-2²* from tomato, which resides on the long arm of chromosome 9 (Ganal et al. 1989; Young et al. 1988). Putative genomic location of *Rpi-vnt1.1* and *Rpi-vnt1.3* allowed for the targeted selection of chromosome-9-specific markers (e.g., TG35 or TG551) to align both linkage groups to potato and tomato chromosome 9. Subsequently, all publicly available *Tm-2²* homologous sequences were aligned to design a set of primers with which we could PCR amplify putatively full-length gene candidates from relevant resistant genotypes. Candidate genes that were genetically closely linked to the *R* loci of interest were cloned in between the promoter and terminator sequences of the recently cloned *Rpi-blb3* gene (Lokossou et al. in press) and targeted for complementation analyses, either through *Agrobacterium* transient transformation assays (ATTA) in *N. benthamiana* or by stable *Agrobacterium*-mediated transformation of the susceptible potato cv. Desiree. Results of transient assays carried out with the initial truncated amplicons in *N. benthamiana* using the appropriate differential isolates suggested that we had indeed cloned two functional *R* genes. However, transgenic Desiree plants transformed with the same gene constructs were fully susceptible to *P. infestans*, whereas those transformed with a 4.3-kb genomic fragment, which was subcloned by Foster and co-workers from a BAC (bacterial artificial chromosome) clone that spanned the *Rpi-vnt1.1* locus (Foster et al. 2009), displayed the expected resistance spectrum. Alignment of the initial truncated amplicon sequences to those of the functional genomic fragment revealed the presence of an additional in-frame ATG start codon 99 nt upstream of the start codon that was initially used as basis for the PCR-based allele-mining experiments, suggesting that the functional *Rpi-vnt1.1* protein had an extended N-terminus of 33 amino acids compared with *Tm-2²*.

Subsequent analysis of the full-length *Rpi-vnt1.1* and *Rpi-vnt1.3* genes from the relevant parental genotypes revealed that *Rpi-vnt1.3* contained an additional 42-nt insertion in the 5' extended region compared with *Rpi-vnt1.1*. Full-length versions of both *Rpi-vnt1.1* and *Rpi-vnt1.3* were shown to confer resistance using transient and stable complementation assays. Although the 5' truncated versions of *Rpi-vnt1.1* and *Rpi-vnt1.3* were not able to confer resistance when expressed as stable transgenes, they were able to induce significant levels of resistance when expressed transiently in ATTA experiments. We speculate that absence of the N-termini of the *R* proteins may be compensated by the relatively high expression inherent to ATTA experiments. *Rpi-vnt1.1* and *Rpi-vnt1.3* belong to the CC-NBS-LRR class of plant *R* proteins. The CC domain, typically containing two or more α helices, which interact to form a super coil structure, is involved in protein-protein interactions (Liu et al. 2006). In the case of *R* proteins, it seems that the CC domain is involved in downstream signaling rather than in recognition (Van der Biezen 2002; Warren et al. 1999). Alignment of several *R* protein sequences recently led to the identification of a conserved EDVID motif among CC-NBS-LRR proteins. This motif was shown to be required for interaction between the CC domain and NBS-ARC or LRR domain (Rairdan et al. 2008). Moreover, this interaction is dependent on a wild-type P-loop motif (Moffett et al. 2002). A model proposed by Takken and co-workers (2006) suggests that the CC domain plays a role as an interactive platform for downstream signaling partners. Upon binding of ATP to the NB domain, a conformational change occurs in the CC domain, which releases the LRR signaling potential. Although the CC domain plays a role in downstream signaling, it cannot trigger the HR on its own. Another model proposed by Moffett and coworkers (2002) suggests that the CC domain may bind to an effector molecule which is released upon conformational changes within the *R* protein leading to the activation of downstream partners. The N-termini of *Rpi-vnt1.1* and *Rpi-vnt1.3* harbor four putative α helices that could interact to form two dimers. The first helix is present in the additional 99 nt upstream of the start codon that corresponds to the start codon of the *Tm-2²* gene. The truncated versions of *Rpi-vnt1.1* and *Rpi-vnt1.3* lack this first helix, thereby destabilizing a possible interaction of the CC domain with the NB or LRR domain leading to less efficient downstream signaling. Overexpression of the truncated protein may override the negative effect of destabilized intramolecular interactions and, thus, have only minor effects on the triggering of the HR upon pathogen attack. This is in line with previous findings that overexpression of functional *R* genes (e.g., *Rx*, *RPM1* and *RPS2*) using the 35S

Fig. 5. Genetic complementation of cv. Desiree using full-length versions of *Rpi-vnt1.1* and *Rpi-vnt1.3* (*Tm2GH-vnt1.1FL* and *Tm2GH-vnt1.3FL*, respectively). Cv. Bintje, cv. Desiree, *Tm2GH-vnt1b*, and *Tm2GH-vnt1.9* were used as susceptible controls and pSLJ21152 (4.3-kb fragment carrying the putative *Rpi-vnt1.1* promoter, open reading frame, and terminator sequences) as resistant control. Primary transformants were challenged with IPO-complex (nonvirulent isolate) and scored 6 days postinoculation. *Tm2GH-vnt1.1FL* primary transformant -16 and *Tm2GH-vnt1.3FL* primary transformant -15 were chosen to illustrate detached leaf assay results. As expected, Bintje, Desiree, *Tm2GH-vnt1b*, and *Tm2GH-vnt1.9* showed late-blight symptoms whereas pSLJ21152 and the full-length primary transformants were fully resistant to IPO-complex.

promoter can lead to cell death responses even in the absence of the pathogen (Belkhadir et al. 2004; Tao et al. 2000).

Complementation analysis of candidate *R* genes is usually done by stable transformation of susceptible potato cultivars. This approach is time consuming, requiring several months to confirm the function of the candidate genes. In the case of an allele-mining approach, one can expect to identify many paralogous candidate resistant genes, which calls for a quick and

efficient complementation assay. Previous studies reported that resistance observed on *N. benthamiana* to *P. infestans* was mediated by the recognition of the elicitor protein INF1 (Kamoun et al. 1998). A transgenic *P. infestans* line, engineered to silence INF1 (Van West et al. 1999), was shown to be virulent on *N. benthamiana*. However, recent screens with a diverse set of *P. infestans* isolates showed that most isolates are, in fact, able to infect *N. benthamiana* (H. Rietman, I. Hein, R. G. F. Visser,

1	MNYCVYKTWAVDSNTKANSTSFLLSSFSYFPFLILTF	<u>RKKKFNEKLKEMAE</u>	ILLTAVINKS	Rpi-vnt1.3	
1		-----		Rpi-vnt1.1	
1	-----	-----	S.....	Tm2 ²	
61	IEIAGNVLFQEGTRLYW	<u>LKEDIDWLQREMRH</u>	IRS YVDNAKAEVGGDSRVKNLLKDIQQL	Rpi-vnt1.3	
47				Rpi-vnt1.1	
14	V.....L.I...K.....		A.....E.	Tm2 ²	
121	AGDV	<u>EDLLDE</u>	FLPKIQSNKFICLKTVSFADEFAM <u>EIEKIKRRVADIDR</u>	VRTTYSITDT Rpi-vnt1.3	
107				Rpi-vnt1.1	
74	D.....	NY...RS.....	V...I.K..N.I..	Tm2 ²	
181	SNNDDC	IPLD	RRRLFLHADETEVIGLEDDFNTLQAKLLDHDLPYGVVSIV	<u>GMPGLGKT</u>	T Rpi-vnt1.3
167				Rpi-vnt1.1	
134	D.....VL.....	I...D...M.....NQ..H.....		Tm2 ²	
241	LAKKLYRHVCHQFEC	SGLVYVSQQPRAGEILHDIKQVGLTEEERKENLENNLRSLKIK		Rpi-vnt1.3	
227				Rpi-vnt1.1	
194	LIRD.....	S...L.....I...QKM.....D.....		Tm2 ²	
301	<u>RYVILLDDI</u>	WDVEIWDDLKLVLPEDCSKIGSRRIITSRNSNVGRYIGGDFS	SIHVLQPLDS	Rpi-vnt1.3	
287				Rpi-vnt1.1	
254	...F...V.....	V.....	ES.L.A...E.	Tm2 ²	
361	EKSFE	LFTKKIFNFVNDN-WANASPDLVNIGRCIVER	<u>CGGIPLAIVV</u>	TAGMLRARGRTEH Rpi-vnt1.3	
347				Rpi-vnt1.1	
314	DDN.S.....	N..G.....	E....	Tm2 ²	
420	AWN	RVLSEMAHKIQDGC	GVLSYNDLPIALRP	<u>CFLY</u>	FGLYPEDHEIRAFDLTNMWIAE Rpi-vnt1.3
406				Rpi-vnt1.1	
374	G..V...A.....	S.....	I.....	Tm2 ²	
480	KLIV	VNTGNGREAESLADDV	LVNLDVSRNLIQVAKRTYDGRIS	SCRIHDLHSLCVDLAKE Rpi-vnt1.3	
466				Rpi-vnt1.1	
434	.F...S..R...D..E.....	L.....N.....		Tm2 ²	
540	SNFFHTEH	<u>Y</u> AFGDPSNVARVRRITFYSDDNAMNEFFHLNPKPMKLRSLFCFTKDRCIFSQ		Rpi-vnt1.3	
526		 <u>N</u>		Rpi-vnt1.1	
494	A.D.....G...L.....	-..V.I...RS...LE...V...A..PS...H		Tm2 ²	
600	MAH	LNFKLLQVLVVMSQKG	YQHVTFPKKIGNMSCLRYVRLEG	AIRVKLPNSIVKLKCLE Rpi-vnt1.3	
586				Rpi-vnt1.1	
553	..YFD.....HT.....	SFQAY..I.S.F...T...L...N.CG.....	TR..	Tm2 ²	
660	TLDIF	HSSSKLP-FGVWESKILRHLCY-----	TEECYCVSFASPF	CRIMPPNNLQTLMW Rpi-vnt1.3	
646				Rpi-vnt1.1	
613	.I...DRR.LIQ.PS.....	H.....RDYQACNS.FSI.SFY.NIYSLH.....		Tm2 ²	
713	VDDK	FCEPRLLHRLINLRLTLCIMDVSGSTIKILSALSPVP	<u>KALEV</u>	LKLRFKNTSEQINL Rpi-vnt1.3	
699			 <u>R</u>	Rpi-vnt1.1	
673	IP...F.....	K.G.LG..N..V.M..IF...L.....S.SSDP....K.		Tm2 ²	
773	SSHP	NIVELGLVGFSAMLLNIEAFPPNLVKLNVLGMLVDG	HLLAVLKKLPKLRILILLWC	Rpi-vnt1.3	
759				Rpi-vnt1.1	
733	..Y.H.AK.H.NVNRT.A..SQS.....	I..T..ANFT..RYI.....TF...K.KMFI.		Tm2 ²	
833	RHDAEKMDLSGDS---	FPQLEVLYIEDAQGLSEVTCMDMSMPKLLKFLVQGP	NISPI	Rpi-vnt1.3	
819				Rpi-vnt1.1	
793	KYNE...A...EANGYS.....	H.HSPN.....T..V.....L.T-.-FH-C		Tm2 ²	
889	SLRV	SERLAKLRISQVL		Rpi-vnt1.3	
875				Rpi-vnt1.1	
849	GISL....K..SK			Tm2 ²	

Fig. 6. Alignment of Rpi-vnt1.1, Rpi-vnt1.3, and Tm2² protein sequences. In the coiled-coil domain, four putative heptad motifs and the conserved motif EDVDID are underlined in bold or italic, respectively. Conserved motifs within the NBS-ARC (nucleotide-binding site apoptosis, *R* gene products, CED-4) domain are underlined in italic. Residues in bold represent the differences between *Rpi-vnt1.1* and *Rpi-vnt1.3* in the leucine-rich repeat domain.

E. A. G. Van der Vossen, and V. G. A. A. Vleeshouwers, *unpublished results*). Previous studies, focused on potato-*Phytophthora* gene-for-gene interactions, showed that co-infiltration of cognate avirulence (*Avr*) and *R* genes in *N. benthamiana* leaves leads to the triggering of an HR typical for incompatible interactions (Bos et al. 2006), indicating that all the necessary *P. infestans* resistance signaling components are present in *N. benthamiana*. We have exploited these findings to develop a transient complementation assay for late-blight *R* genes using ATTA in combination with DLA. Despite the successful use of the transient assay in the cloning of the functional *Rpi-blb1* orthologs *Rpi-sto1* and *Rpi-pta1* (Vleeshouwers et al. 2008), the results obtained with the truncated *Rpi-vnt1.1* and *Rpi-vnt1.3* genes indicate that one needs to be careful with the interpretation of the results, and that stable transformation of a susceptible potato cultivar remains the ultimate functional assay of a gene.

Chromosomes 9 of tomato and potato seem to be hot spots for resistance because several *R* genes, conferring resistance to a broad range of pathogens, are located on this chromosome. In tomato, in addition to *Tm-2²*, four other *R* genes have been mapped on chromosome 9; *Sw5* at the distal end of the long arm, conferring resistance to *Tomato spotted wilt virus* (Spasova et al. 2001); *Ve1* and *Ve2*, conferring resistance to *Verticillium dahliae* (Simko et al. 2004); and *Frl*, conferring

resistance to *Clavibacter michiganensis* subsp. *michiganensis* (Vakalounakis et al. 1997). Interestingly, in tomato, three major QTL for resistance to *P. infestans* have been described, one of which, *Ph3*, is located at the distal end of the long arm of chromosome 9 (Chunwongse and Black 2002). In potato, *Nx-Phu*, a potato gene for hypersensitive resistance to *Potato virus X* (Tommiska et al. 1998); the late blight *R* genes *Rpi-mcq1* (Smilde et al. 2005); and *Rpi-phu1* (Sliwka et al. 2006) reside on chromosome 9. In the current study, we mapped *Tm2GHs* at the distal end of the long arm of chromosome 9, a region that coincides with the *Rpi-mcq1* locus (Fig. 6), suggesting that these two genes are likely to be *Tm2GH*. *Rpi-phu1* is also likely to be *Tm2GH* because it was mapped in the region harboring *Tm2GH* clusters. In addition to the above-described *R* loci, several QTL have also been described on chromosome 9 of potato that confer foliage resistance to *P. infestans* (*Pin9a*), resistance to the root cyst nematode *Globodera pallida* (*Gpa6*), and tuber and leaf resistance to *Erwinia carotovora* subsp. *atroseptica* (*Eca9A*) (Gebhardt and Valkonen 2001). To what extent *Tm2GHs* are also involved in quantitative resistance remains unclear.

Alignment of the *Rpi-vnt1.1* and *Rpi-vnt1.3* protein sequences to those of the currently cloned genes *R1*, *R3a*, *Rpi-blb1*, *Rpi-blb2*, and *Rpi-blb3* (Ballvora et al. 2002; Huang et al. 2005) (Lokossou et al. in press) revealed very little homol-

Fig. 7. Relative genetic positions of *Tm2GH* in potato and tomato. **A**, Schematic linkage maps of potato and tomato showing five resistance gene clusters. **B**, Ultra Dense potato genetic map of chromosome 9 divided in BINs from the parents SH83-92-488 (SH) and RH89-039-16 (RH). The thick vertical bars show the location of *Tm2GHs* belonging to the non-NBS3B-like group in the genome of SH and RH. The distal end of the long arm of the chromosome 9 from SH and RH harbors *Tm2GHs* in BINs 78-82 and BINs 77-81, respectively. This region coincides with the late-blight-resistant loci *Rpi-mcq1* and *Ph-3* from potato and tomato, respectively.

ogy to those genes, although all belong to the CC-NBS-LRR class of plant *R* genes. This is the first time that *Tm2GH* are shown to be involved in late-blight resistance. Alignment of the protein sequences of the chromosome-9-derived *R* genes (*Tm-2²*, *Sw5a*, *Sw5b*, *Ve1*, and *Ve2*) suggests the presence of three distinct gene families, *Tm2*-like, *Sw5*-like, and *Ve*-like. The *Ve* genes are located on the short arm of chromosome 9 whereas the *Sw5* genes are located at the distal end of the long arm (Fig. 6). *Tm-2²* and the cloned or mapped *Rpi* genes reside at three *Tm2GH* clusters that are spread across the long arm of chromosome 9 (Fig. 6), suggesting that *Tm2GH* is the predominant gene family on this chromosome arm. The overall homology between the identified *Tm2GH* ranges between 74 and 99.8% amino acid identity, suggesting that they have a common origin and that they have arisen through gene duplication events. Gene duplication and sequence exchange between *RGH* are major mechanisms that shape *R* gene diversity in plants (Kuang and Woo 2004; Meyers et al. 1999; Michelmore and Meyers 1998). Comparison of the protein sequence of *Tm-2²* with those of *Rpi-vnt1.1* and *Rpi-vnt1.3* revealed a higher homology within the CC and NB-ARC domains (86.5%) than the LRR domain (62%), which is in line with the fact that they confer resistance to completely different pathogens, ToMV and *P. infestans*, respectively, and that the LRR domain primarily determines recognition specificity and, thus, is subject to diversifying selection (Ellis et al. 2000). The functions of the CC and NBS domains, on the other hand, are likely to be more conserved, encompassing the fine tuning of intramolecular interactions (Moffett et al. 2002) and the regulation of downstream signaling (Belkadir et al. 2004; Van der Biezen 2002; Warren et al. 1999).

The rapid breakdown of the first introgressed *Rpi* genes from *S. demissum* previously stimulated breeders to reconsider their breeding goals and, subsequently, efforts toward improving late-blight resistance were focused on increasing partial resistance by using race-nonspecific sources of resistance. However, under long-day conditions, breeders using this strategy have achieved little progress, the major drawback being the correlation between foliage resistance and late foliage maturity. Nevertheless, we anticipate that breeding for late-blight resistance in potato aimed at substantially contributing to disease management requires, by one way or another, the deployment of *Rpi* genes. As more and more *Rpi* genes are identified and cloned, the chances increase that new *Rpi* genes reside at known and well-characterized loci, enabling the use of comparative genomics and, thus, the development of efficient allel mining strategies. Moreover, ongoing potato and tomato genome sequencing projects by international consortia are providing a (complete) survey of the distribution of *R* gene clusters in the family *Solanaceae*, enabling even faster cloning of *Rpi* genes. Challenges that remain are how to predict *Rpi* gene durability and how to introduce durable combinations of *Rpi* genes into existing and future cultivars in the most efficient and sustainable manner. We believe that knowledge of effector diversity may provide clues about the putative durability of *Rpi* genes (Birch et al. 2008; Vleeshouwers et al. 2008). The recent discovery of a common RXLR motif in oomycete Avr effector proteins (Birch et al. 2006; Rehmany et al. 2005) promises to accelerate the discovery and functional characterization of late-blight *Rpi* genes and of *P. infestans* Avr genes and, thus, the engineering of durable late-blight-resistant potato cultivars in the future. Efficient stacking of *Rpi* genes from one or several species is essential; however, in practice, this will enlarge the linkage drag problems considerably. Their introduction by genetic modification is a much more efficient way to improve resistance in one step and in a short period. It can even be applied to existing cultivars with a long history of safe use. Cur-

rently, *Rpi* genes of natural origin derived from sexually compatible species, so-called cisgenes (Schouten et al. 2006), can be introduced using marker-free transformation systems (Vetten et al. 2003), leading to cisgenic plants with only the gene or genes of interest and without linkage drag (Jacobsen and Schouten 2007). It implies an environmental benefit and will, therefore, hopefully meet with consumer acceptance.

MATERIALS AND METHODS

Plant material.

Accessions of *S. venturii* were provided by the Centre of Genetic Resources (CGN) in Wageningen, The Netherlands. Following screening with *P. infestans*, resistant genotypes vnt367-1 and vnt367-1 from accessions CGN18108 and CGN18000, respectively, were used to generate inter- or intra-specific mapping populations. The *Rpi-vnt1.1* mapping population 7698 was generated by crossing vnt7014-9 (resistant) (vnt367-1 [resistant] × vnt366-8 [susceptible]) × nrs735-2 (susceptible). The *Rpi-vnt1.3* mapping population 7663 was generated by crossing the resistant plant vnt365-1 (resistant) × nrs735-2 (susceptible). The genes observed in CGN18108 and CGN18000 (initially called *Rpi-okal* and *Rpi-nrs1*, respectively) (*unpublished results*) were named after the species *S. okadae* and *S. neorossi*. However, DNA fingerprinting of a wide range of wild *Solanum* spp. showed that the GenBank accessions CGN18108 and CGN18000 clustered with other accessions of *S. venturii* (Jacobs 2008; Jacobs et al. 2008). Morphological inspection of the CGN18108 and CGN18000 accessions confirmed that the labels *S. okadae* and *S. neorossi* should indeed be replaced with the label *S. venturii*. Furthermore, all available GenBank accessions of *S. okadae* and *S. neorossi* were susceptible and did not amplify a PCR product with our *Rpi-vnt1.1*- or *-1.3*-specific primers (M. A. Pel, *unpublished results*).

Disease assays and agroinfiltration.

DLA on the *Solanum* spp. were carried out as described by Vleeshouwers and associates (1999). Leaves were inoculated with 10-μl droplets of inoculum (5 × 10⁴ zoospores/ml) on the underside and incubated at 15°C for 6 days in a climate chamber with a photoperiod of 16 and 8 h, day and night, respectively. At 6 days postinoculation, leaves showing sporulation were scored as susceptible whereas leaves showing no symptoms or necrotic lesions were scored as resistant.

ATTA were performed in *N. benthamiana* followed by DLA using appropriate *P. infestans* isolates. Four-week-old plants were infiltrated with a solution of *A. tumefaciens* COR308 (Hamilton and Frary 1996) harboring putative *R* gene candidates. Agroinfiltration of the lower side of *N. benthamiana* leaves was carried according to Van der Hoorn and associates (2000) using an optical density at 600 nm of 0.1. Two days post-infiltration, a DLA was performed as mentioned above. The phenotyping was done between 4 and 7 days postinoculation.

In ATTA assays, for each construct tested, three leaves of three different *N. benthamiana* plants were infiltrated with either *Tm2GH-vnt1b* (truncated *Rpi-vnt1.1* candidate gene), *Tm2GH-vnt1.9* (truncated *Rpi-vnt1.3* candidate gene), *Rpi-sto1* (resistant control), or *abpt-RGH-a* (nonfunctional resistance gene). Assays were repeated three times. Infection efficiency was quantified by scoring 36 inoculation spots for each infiltrated constructs (three times one plant with three infiltrated leaves each harboring four inoculated spots).

Marker development.

Candidate gene markers were developed through NBS profiling as described by Van der Linden and associates (2004). Templates were generated by restriction digestion of genomic

DNA using the restriction enzymes *MseI*, *HaeIII*, *AluI*, *RsaI*, or *TaqI*. Adapters were ligated to restriction fragments. PCR fragments were generated by radioactive-labeled primers (nbs1, nbs2, nbs3, nbs5a6, or nbs9) designed on conserved domains of the NBS domain (P-loop, Kinase-2 and GLPL motifs) (Calenge et al. 2005; Syed and Srensen 2006). Additional markers from appropriate chromosomal positions were selected from the SGN database and subsequently developed into polymorphic markers in each of the relevant mapping populations.

PCR amplification of candidate *R* genes.

Long-range PCR with Taq-polymerase or *Pfu* Turbo polymerase in a 50- μ l reaction mixture was prepared containing 50 ng of genomic DNA, 1 μ l of the forward primer (10 μ M), 1 μ l of the reverse primer (10 μ M), 0.8 μ l of dNTPs (5 mM each), 5 μ l of 10 $\times$ buffer, 5 units of Taq polymerase (Perkin Elmer, Norwalk, CT, U.S.A.), or 1 μ l of *Pfu* Turbo (Invitrogen, San Diego, CA, U.S.A.). The following PCR program was used: 94°C for 3 min, 94°C for 30 s, 55°C for 30 s, 72°C for 4 min, and 72°C for 5 min during 29 cycles.

Genome walking.

Marker sequences were extended by cloning flanking DNA fragments with the Clontech Genome Walker kit (Clontech Labs, Inc., Palo Alto, CA, U.S.A.) according to the manufacturer's instructions using a blunt adapter, comprising the complementary sequences 5'GTAATACGACTCACTATAGGGCA CGCGTGGTTCGACGGCCCGGGCTGGA and 5'PO4-TCCA GCCC and the adapter-specific primers AP1 (TAATACGACT CACTATAGGGC) and AP2 (5'ACTATAGGGCACGCGTGT GT). A simultaneous restriction ligation was performed followed by two rounds of PCR. A 50- μ l restriction-ligation (RL) mixture was prepared containing 250 ng of genomic DNA, 5 units of blunt cutting enzyme (*Bsh1236I*, *AluI*, *DpnI*, *HaeIII*, *RsaI*, *HincII*, *DraI*, *ScaI*, *HpaI*, or *SspI*), 1 μ l of genome walker adapter (25 μ M), 10 mM ATP, 10 μ l of 5 $\times$ RL buffer, and 1 unit of T4 DNA ligase (1 U/ μ l) (Invitrogen). The digestion mix was incubated at 37°C for 3 h. Samples were diluted 50 times prior to PCR. For the first PCR round, a 20- μ l reaction mixture was prepared containing 5 μ l of diluted RL DNA, 0.6 μ l of specific forward primer 1 (10 μ M), 0.6 μ l of AP1 (10 μ M), 0.8 μ l of dNTPs (5 mM each), 2 μ l of 10 $\times$ buffer (Perkin Elmer), and 5 units of Taq polymerase (Perkin Elmer). The first PCR was performed using the following cycle program: 30 s at 94°C as the denaturation step, 30 s at 56°C as the annealing step, and 60 s at 72°C as the extension step; 35 cycles were performed. A second PCR using the same conditions as the first one was performed using specific primer 2 and AP2 and 5 μ l of 50 $\times$ diluted product from the first PCR. The second PCR product (5 μ l) was checked on gel (1% agarose) and the largest amplicons were cloned into the pGEM-T Easy Vector from Promega (Madison, WI, U.S.A.) and sequenced.

Gateway cloning of candidate *R* genes into a binary expression vector.

The Gateway cloning technique was used according to the manufacturer's instructions to efficiently clone candidate genes together with appropriate promoter and terminator sequences into the binary Gateway vector pKGW-MGW. In plasmid pKGW, the gateway cassette was exchanged against a multiple gateway cassette amplified from pDESTr4r3, resulting in pKGW-MGW. In this study, we used the promoter and terminator of *Rpi-blb3* (Lokossou et al. in press), which were cloned into the Gateway pDONR vectors pDONRP4P1R and pDONRP2RP3, respectively, generating pENTR-B1b3P and pENTR-B1b3T. PCR amplicons generated with *Pfu* Turbo polymerase were cloned into pDONR221 generating pENTR-

RGH clones and, subsequently, cloned together with the *Rpi-blb3* promoter and terminator fragments into pKGW-MGW using the multiple Gateway cloning kit (Invitrogen). The pENTR clones were made by carrying out a BP-Reaction II overnight. DH5 α competent cells (Invitrogen) were transformed by heat shock with 5 μ l of the BP Reaction II mixture. Cells were selected on Luria-Bertani (LB) medium containing of kanamycin at 50 mg/ml. Colonies were checked for the presence of the relevant inserts by colony PCR. DNA of appropriate pENTR clones was extracted from *Escherichia coli* and used to perform a multiple Gateway LR cloning reaction to generate the final binary expression clones (pVNT1 and pNRS1). DH5 α competent cells (Invitrogen) were transformed by heat shock with 5 μ l of the LR reaction mixture. Cells were selected on LB medium containing spectinomycin at 100 mg/ml. Colonies were checked by PCR for the presence of the correct inserts. Positive colonies were grown overnight in LB medium supplemented with spectinomycin at 100 mg/ml to extract the final expression vector. The final expression vector was transferred to *A. tumefaciens* COR308 through electroporation. Colonies were selected on LB medium supplemented with spectinomycin at 100 mg/ml and tetracycline at 12.5 mg/ml overnight at 30°C.

Stable transformation.

Binary vectors carrying *Tm2GH-vnt1b*, *Tm2GH-vnt1.9*, pSLJ21152, *Tm2GH-vnt1.1FL*, or *Tm2GH-vnt1.3FL* were used for *Agrobacterium*-mediated transformation in strain COR308. Internodia with a size of 2 to 5 mm were cut from explants of cv. Desiree and transferred to R3B-medium plates containing two sterile filter papers. The next day, internodia were incubated for 5 to 10 min in the bacteria solution, dried, transferred back to R3B plates, and placed at 24°C with a light period of 16 h. Two days later, internodia were transferred to selective medium containing kanamycin (100 mg/liter), zeatine (1 mg/liter), claforan (200 mg/liter), and vancomycin (200 mg/liter). Every 2 weeks, internodia were transferred to fresh selective medium. Green shoots emerging from calli were harvested and transferred to MS30 medium supplemented with kanamycin (100 mg/liter) to regenerate plantlets.

5' RACE.

The GeneRacer Kit from Invitrogen was used according to the manufacturer's instructions to determine the 5' terminal structure of *Rpi-vnt1.1* and *Rpi-vnt1.3*. Two gene-specific primers (GSP1-5race and GSP2-5race) (Table 3) were designed to perform a nested PCR using cDNA template from relevant parental genotypes and transgenic plants and High Fidelity Platinum *Taq* DNA Polymerase (HT Biotechnology Ltd., Cambridge). PCR products were cloned into the pGEM-T Easy Vector (Promega) and sequenced.

Sequencing.

Cloned fragments or PCR products generated with either Taq polymerase (Perkin Elmer) or *Pfu* Turbo polymerase (Invitrogen) were sequenced as follows: 10 μ l of sequencing reaction mixture was made using 5 μ l of PCR product or 5 ng of plasmid, 3 μ l of buffer, 1 μ l of DETT (Amersham), and 1 μ l of forward or reverse primer. The PCR program used was 25 cycles of 94°C for 20 s, 50°C for 15 s, and 60°C for 1 min. The sequences were generated on ABI 3730XL sequencers.

Mapping of *Tm2GHs* in the ultradense genetic map of potato.

The Ultra Dense genetic map of potato, which was previously made with a mapping population consisting of 130 F1 progeny derived from SH $\times$ RH (Roupe van der Voort et al.

1997), was used to map *Tm2GH*. Genomic DNA from the parents (SH and RH) were PCR amplified using the primers ATG-Tm2-F and Rev-C (Tables 2 and 3). Amplification products were digested with restriction enzymes to detect polymorphisms between the parents. A subpopulation of 50 F1 individuals was subsequently analyzed through PCR amplification and appropriate restriction digestion. PCR reaction mixtures (20 µl) contained 50 ng of genomic DNA, 1 µl of the forward primer (10 µM), 1 µl of the reverse primer (10 µM), 0.8 µl of dNTPs (5 mM each), 5 µl of 10× buffer, and 5 units of Taq polymerase (Perkin Elmer). The following PCR program was used: 94°C for 3 min, 94°C for 30 s, 55°C for 30 s, 72°C for 4 min, and 72°C for 5 min during 29 cycles. PCR product (5 µl) was digested at the required temperature for 3 h with the appropriate restriction enzymes. Segregating polymorphisms were mapped in the ultradense potato map relative to 10,000 amplified fragment length polymorphism markers.

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AUTHOR-RECOMMENDED INTERNET RESOURCES

- Andreas Untergassen Lab's BP-Reaction protocol webpage:
www.untergassen.com/lab/protocols/bp_gateway_reaction_ii_v1_0.htm
- European Union project FAIR5-PL97-3565 ultra-dense genetic map:
www.plantbreeding.wur.nl/potatomap