

A novel approach to locate *Phytophthora infestans* resistance genes on the potato genetic map

Mirjam M. J. Jacobs · Ben Vosman ·
Vivianne G. A. A. Vleeshouwers · Richard G. F. Visser ·
Betty Henken · Ronald G. van den Berg

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Abstract Mapping resistance genes is usually accomplished by phenotyping a segregating population for the resistance trait and genotyping it using a large number of markers. Most resistance genes are of the NBS-LRR type, of which an increasing number is sequenced. These genes and their analogs (RGAs) are often organized in clusters. Clusters tend to be rather homogenous, viz. containing genes that show high sequence similarity with each other. From many of these clusters the map position is known. In this study we present and test a novel method to quickly identify to which cluster a new resistance gene belongs and

to produce markers that can be used for introgression breeding. We used NBS profiling to identify markers in bulked DNA samples prepared from resistant and susceptible genotypes of small segregating populations. Markers co-segregating with resistance can be tested on individual plants and directly used for breeding. To identify the resistance gene cluster a gene belongs to, the fragments were sequenced and the sequences analyzed using bioinformatics tools. Putative map positions arising from this analysis were validated using markers mapped in the segregating population. The versatility of the approach is demonstrated with a number of populations derived from wild *Solanum* species segregating for *P. infestans* resistance. Newly identified *P. infestans* resistance genes originating from *S. verrucosum*, *S. schenckii*, and *S. capsicibaccatum* could be mapped to potato chromosomes 6, 4, and 11, respectively.

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M. M. J. Jacobs · R. G. van den Berg
Biosystematics Group, Wageningen University and Research
Centre, Generaal Foulkesweg 37, 6703 BL Wageningen,
The Netherlands
e-mail: m.jacobs@senternovem.nl

R. G. van den Berg
e-mail: ronald.vandenberg@wur.nl

M. M. J. Jacobs · B. Vosman (✉) · V. G. A. A. Vleeshouwers ·
R. G. F. Visser · B. Henken
Wageningen UR Plant Breeding, P.O. Box 16,
6700 AA Wageningen, The Netherlands
e-mail: ben.vosman@wur.nl

V. G. A. A. Vleeshouwers
e-mail: vivianne.vleeshouwers@wur.nl

R. G. F. Visser
e-mail: richard.visser@wur.nl

B. Henken
e-mail: betty.henken@wur.nl

M. M. J. Jacobs · B. Vosman · V. G. A. A. Vleeshouwers ·
R. G. F. Visser · B. Henken · R. G. van den Berg
Centre for BioSystems Genomics,
P.O. Box 98, 6700 AB Wageningen, The Netherlands

Introduction

Plants are attacked by a wide range of pathogens including viruses, bacteria, oomycetes, fungi, nematodes, and insects. They have evolved passive and active ways to defend themselves against these attackers. One of the active defense systems is a type of immunity that is described by the gene for gene resistance theory, which was developed by Flor in the 1940s. It considers the gene causing resistance, the R gene in the host, to be complementary to an Avr (avirulence) gene in the pathogen (Flor 1942, 1971). To date, more than 90 resistance (R) genes have been identified in various plants, by a wide variety of methods including map-based cloning, transposon tagging, and similarity-based DNA library screening (Ingvarsdson et al. 2008). Most R genes can be assigned to one of the five major classes

of R genes (Dangl and Jones 2001). The largest of these classes contain genes that encode proteins with a nucleotide binding site and a leucine-rich repeat region (the so called NBS-LRR genes). NBS-LRR resistance genes and their analogs (RGAs) are numerous in plant genomes and are often organized in clusters (Michelmore and Meyers 1998; AGI 2000). Many of the R genes in *Solanum* seem to be positioned in relatively few clusters (Gebhardt et al. 1991; Tanksley et al. 1992; Bakker et al. 2003).

The common approach to map resistance genes is to construct a mapping population derived from a susceptible and a resistant parent, phenotype the offspring, and then analyze the offspring with molecular markers. As many resistance traits turned out to be controlled by a single gene, more efficient methods have been developed to facilitate the search for markers linked to these genes. Bulk segregant analysis is a method for efficiently identifying markers linked to a specific trait. Two pooled DNA samples of individuals from a segregating population with contrasting phenotypes resulting from a single cross are compared. Michelmore et al. (1991) showed that this approach works well to rapidly identify RAPD and RFLP markers linked to the trait of interest.

The NBS region of (NBS-LRR) R genes and RGAs contains highly conserved common motifs like the P-loop, the kinase-2 motif, and the GLPL motif (Meyers et al. 1999, 2003; Monosi et al. 2004). These conserved motifs within the NBS-LRR genes have been used successfully to sequence parts of NBS regions from various plant species (Collins et al. 1998; Pflieger et al. 1999; Zhang et al. 2007). Van der Linden et al. (2004) developed a method for efficiently tagging NBS-LRR type of resistance genes and their analogs called NBS profiling. NBS profiling is a PCR-based method that makes use of primers that target different conserved motifs in the NBS domain. It produces a DNA profile that is highly enriched for R genes and RGAs. Studies in apple (Calenge et al. 2005) and in potato, tomato, barley, and lettuce (van der Linden et al. 2004) show that NBS profiling produces markers that are tightly linked to R genes and R-gene clusters.

Late blight caused by the oomycete *Phytophthora infestans* is one of the most important and devastating diseases in potato. Currently, late blight is mainly controlled by a combination of disease management strategies, relying heavily on the use of fungicides (Fry 2008). High disease management costs, environmental concern, and the threat of promoting the evolution of resistant populations stimulated the search for R genes that can be used in breeding programs to create resistant cultivars. In the past, 11 late blight resistance genes from the wild potato species *Solanum demissum* (Gebhardt and Valkonen 2001) were introduced into cultivated potato. As some of the resistances conferred by these R genes were quickly overcome by the

pathogen (Wastie 1991) the focus of breeders and scientist moved toward germplasm with partial or quantitative resistance (van der Vossen et al. 2005; Fry 2008). More recently, the interest in finding new R genes has increased again. The presence of R genes conferring resistance against *P. infestans* in other wild potato species than *S. demissum* was investigated as well. Resistance against *P. infestans* conferred by R genes has been found in *S. pinnatisectum* (Kuhl et al. 2001), *S. bulbocastanum* (Naess et al. 2000; Song et al. 2003; van der Vossen et al. 2003, 2005; Park et al. 2005a), *S. berthaultii* (Ewing et al. 2000; Rauscher et al. 2006), *S. microdontum* (Sandbrink et al. 2000; Tan et al. 2008), *S. mochiquense* (Smilde et al. 2005), *S. paucisectum* (Villamon et al. 2005), *S. phureja* (Śliwka et al. 2006) *S. venturii* (Pel et al. 2009, Foster et al. 2009), and *S. stoloniferum* (Wang et al. 2008a).

There are still many other wild species that have not been tested yet for the presence of R genes against *P. infestans*. In the present study, we searched for new *P. infestans* R genes and markers in the wild potato species *S. verrucosum*, *S. schenckii*, and *S. capsicibaccatum*. We present and test a novel approach to quickly identify at which chromosome/chromosomal region the targeted resistance gene is located and to obtain markers that can be used for introgression breeding. The novelty of the approach is in the combination of small size segregating populations in combination with NBS profiling techniques and bioinformatics to quickly retrieve a putative map position of the segregating gene. We illustrate this approach by describing three cases using wild *Solanum* populations that are segregating for *P. infestans* resistance.

Materials and methods

Plant material

The plant material used as parents for the segregating populations was selected from a large screen of around 1,000 accessions of mainly wild *Solanum* section *Petota* germplasm. The evaluated material was described by Jacobs et al. (2008). The following segregating populations were used in this study and details on the progeny can be found in Table 1. The ver03-392 and ver03-394 are very similar BC2 populations, both *S. verrucosum* derived mothers (ver00-3228 and ver00-3229) are late blight resistant clones derived from the same cross (ver 98-28-2 × AR 93-2090) and they were crossed with the same susceptible male parent. The ver 98-28-2 clone is derived from CGN 17772 (BGRC8255). The population snk7458 was a F1 population based on a resistant individual *S. schenckii* GLKS 30659 crossed with a sensitive parent *S. brachycarpum* CGN 18347. Population cap7358 was a F1 population derived

Table 1 Segregating populations used in this study

Population	Parents	Population size/resistance score				Bulk size	
		Total	R ^a	Q ^b	S ^c	R ^a	S ^c
ver03-392	<i>S. verrucosum</i> ver00-3228 × AR95-2172	13	6	4	3	5	3
ver03-394	<i>S. verrucosum</i> ver00-3229 × AR95-2172	17	8	4	5	7	5
snk7458	<i>S. schenckii</i> GLKS 30659 × <i>S. brachycarpum</i> CGN 18347	49	39	5	5	6	4
cap7358	<i>S. capsicibaccatum</i> CGN 22388 × <i>S. circaeifolium</i> CGN 18133	52	34	0	18	10	10

^a R resistant

^b Q quantitative, intermediate phenotype could not be scored unequivocally

^c S susceptible

from a cross with resistant parent *S. capsicibaccatum* CGN 22388 and susceptible parent *S. circaeifolium* CGN 18133.

Phytophthora infestans isolate and disease testing

The aggressive and complex *P. infestans* isolate 90128 (race 1.3.4.7.8.11), kindly provided by Prof. Francine Govers (Laboratory of Phytopathology, Wageningen University) was cultured on cultivar Bintje leaves or on rye sucrose medium as described previously (Vleeshouwers et al. 1999). For disease testing, leaves from 8 to 10-week-old plants were used. The third and fourth fully stretched leaves (counted from the top) were detached, and placed in water-saturated florists foam. For each plant genotype, two replicates were inoculated on five leaflets of one leaf, resulting in 10 replicates per genotype. The leaves were inoculated with a zoospore suspension of 50,000 spores/ml and incubated in humid trays. After 6 days, the leaves were examined for occurrence of sporulation and the lesions sizes (LS) measured. The measurements were normalized for the lesion size using the reference cultivar Bintje.

DNA extractions and NBS profiling

After 7 to 8 weeks of growing, young plant leaves were harvested for DNA extraction. DNA was extracted according to Fulton et al. (1995). NBS profiling was performed as described by van der Linden et al. (2004), with some minor modifications. The protocol of NBS profiling involves three steps: (1) restriction enzyme digestion of genomic DNA and the ligation of adapters (which in our experiments was done in one and the same incubation step, except when the enzyme *TaqI* was used), (2) selective amplification of fragments containing an NBS motif using a degenerated primer for the conserved domains, and (3) polyacrylamide gel electrophoresis of the amplified fragments.

The following 5 restriction enzymes: *MseI*, *TaqI*, *RsaI*, *AluI*, and *HaeIII*, were used in combination with 5 NBS primers: NBS1, NBS2, NBS3, NBS5a6, and NBS9 (van der

Linden et al. 2004; Wang et al. 2008b; Mantovani et al. 2006; Brugmans et al. 2008), resulting in 25 primer–enzyme combinations. NBS profiling was carried out first on the parents and on bulks of pooled resistant and susceptible plants from the population of interest. When this first round produced polymorphic bands between the parents and between the bulks, another round of NBS profiling was carried out on the DNA of the parents, the bulks, and all individuals separately that constituted the resistant and susceptible bulk using only the primer–enzymes combinations that produced polymorphic bands. In the two small *S. verrucosum* populations ver03-392 and ver03-394, only samples from 5 resistant and 3 susceptible and 7 resistant and 5 susceptible plants, respectively, could be scored reliably for *P. infestans* resistance.

Sequence analysis of polymorphic bands

To determine the sequence of a polymorphic NBS marker, the band was excised from the gel and re-amplified with the same primers that initially produced the band. The PCR conditions were identical to the first PCR of the NBS profiling protocol. Only bands that were clearly separated from surrounding bands were considered. In the case of population 7358 it was necessary to clone the band first because direct sequencing showed that the band consisted of a mixture of two fragments. For this, the PCR products were ligated into the pGEM-T easy Vector System (Promega). Ligation mixtures were transformed into *E. coli* DH5 α , as recommended by the supplier (Invitrogen). Colonies containing a plasmid with insert were used for colony PCR. Clones were sequenced using vector M13 primers. For populations ver03-392, ver03-394, and snk7458 the bands were directly sequenced following reamplification. Each fragment was sequenced from both sides with the NBS profiling primers using the Big Dye Terminator Kit on an ABI 3700 automated sequencer (Applied Biosystems, USA). DNA sequences were analyzed using DNASTar (Lasergene, Madison, WI, USA). The obtained sequences were compared

to the NCBI nucleotide database (<http://www.ncbi.nlm.nih.gov/>), by using the discontinuous megablast option of the BLASTN suite (Altschul et al. 1997) from NCBI. The similarity scores with sequences found in the NCBI database were evaluated taking into account the E value. The E value is also dependent on the length of the query sequence that can be blasted to a certain sequence in the database. The shorter the sequence, the higher the possibility that the result is due by chance. A similarity was defined as a good hit if it showed a combination of a similarity (identity) score of 75% or higher plus a small expected value of 1.00E-25 or smaller. The sequences have been submitted to Genbank and are available under the accession numbers GU060647–GU060661 (see also Table 3).

Confirmation of position with PCR-based primers

To verify the putative map positions for NBS profiling markers deduced from the BLAST analysis, we used flanking markers (mainly CAPS). For each population, flanking markers were tested on the parents and all individuals of the cross that could be scored as clearly resistant or susceptible. Many CAPS and SCAR markers were tested, but only few showed polymorphisms in the offspring. The details on the primers that were successfully used are given in Table 4. To amplify the samples with PCR marker Th21, approximately 10 ng of genomic DNA was mixed in a total volume of 20 μ l containing (final concentration per reaction) 1 \times PCR buffer, 0.2 mM mixture of all dNTPs, 0.1 μ M of each primer and 0.1 unit Taq DNA polymerase (Promega). The following PCR protocol was used: a first step of 3 min at 96°C, followed by 30 cycles of 0.5 min at 96°C, 0.5 min at 56°C, and 1 min at 72°C, concluding with 10 min at 72°C. With the CAPS markers CP58 and CD67 a slightly different mixture and protocol was used. Approximately 10 ng of DNA was mixed in a total volume of 25 μ l containing 1 \times PCR buffer, 0.12 mM dNTPs, 0.05 μ M from each primer, and 0.1 unit Super Taq DNA polymerase. The following PCR protocol was used: starting with 4 min at 94°C, followed by 35 cycles of 0.5 min at 94°C, 0.5 min of 58°C, and 1.5 min at 72°C. At the end of the protocol, 6 min at 72°C were programmed. The presence of PCR products of the correct length was evaluated on a 1.0% agarose gel.

Results

Segregation analysis for resistance to *P. infestans*

To analyze whether R genes segregate in the generated populations, the offspring and parents were inoculated with *P. infestans* and assessed for resistance. From the ver03-392 and ver03-394 populations only very few progeny

plants were available. Still, for both populations a segregation for resistance could be detected in repeated experiments, although not all individuals could be scored as clearly resistant or susceptible (Table 1). Population snk7458 also segregated for resistance to *P. infestans* isolate 90128 yet there were far more resistant than susceptible individuals, i.e. 39 and 5, respectively, and in addition a few genotypes with intermediate phenotypes. For cap7358, a clear segregation between resistance and susceptibility was evident, and no intermediate phenotypes were observed. From all populations, subsets of genotypes with reliable phenotypes could now be assembled as resistant and susceptible bulks (Table 1), for further analysis by NBS profiling.

NBS profiling

The populations differed strongly in the number of polymorphic bands in the bulks that showed co-segregation with resistance, ranging from 1 in population cap7358 to 33 in the ver03-392 population (Table 2). For populations snk7458, cap7358, and ver03-394 all the primer/enzyme combinations that produced polymorphic bands were tested on the individuals of the bulks. In the population ver03-392, only a selection of primer/enzyme combinations producing polymorphic bands in the bulks was tested on the individuals. An example of an NBS gel for parents, bulks, and individuals is given in Fig. 1. Not all putative polymorphisms observed in the bulks were validated in the individuals (see Table 2). Bands that were found and confirmed as co-segregating in the second round of NBS profiling on individuals of the bulks were bands in coupling phase, e.g. co-segregating with the resistant phenotype, or in repulsion phase, e.g. co-segregating with the susceptible phenotype. All NBS markers that were studied in the individuals of the bulks co-segregated with resistance or susceptibility as

Table 2 Summarized results of NBS profiling on bulks and individuals

Population	Polymorphic bands (bulks)	Bands coupling phase (individuals)	Bands repulsion phase (individuals)
ver03-392	33	8/18	2/5
ver03-394	19	12/13	4/6
snk7458	10	4/5	5/5
cap7358	1	1/1	0/0

Bands in coupling or repulsion phase refers to the number of polymorphic bands that could be reproduced in the individuals that constituted the bulk. For population ver03-392 not all the primer–enzyme combinations that gave polymorphisms in the bulks were tested in the individual NBS profiling step. 8/18 in this case means that 8 out of the 18 bands that were found to be in coupling phase in the NBS profiling on the bulks, could be reproduced in NBS profiling on the individual plants

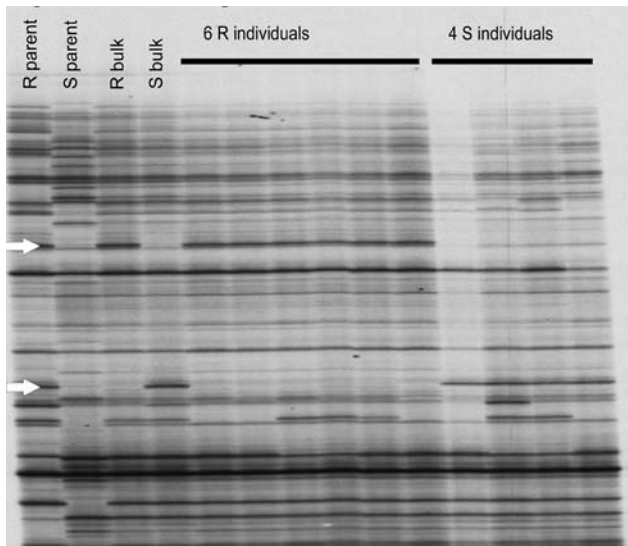


Fig. 1 An example of a part of a NBS profiling gel. This figure shows part of the NBS profiling gel of population snk7458 using NBS2 and *Mse*I. The arrows indicate the segregating NBS profiling bands. The upper arrow points to a band in coupling phase, the lower arrow points to a band in repulsion phase

expected, except for the NBS markers ver03-394_9H1, ver03-394_9R1, and ver03-394_9R2 which show 1 (identical) recombinant resistant plant (out of 7 resistant plants) that does not have the specific NBS fragment.

Identification of NBS bands and deduction of map positions

The bands co-segregating with the resistance phenotype in the individuals were excised from the gels and sequenced to determine their identity. Sequences obtained were compared to the NCBI database using BLAST analysis. Most of these sequences gave hits with NBS-related sequences, so these sequences we regarded as RGAs. For a few sequences there were no hits at all with NCBI database. The best hits (high identity score and low E value) of these sequences to *Solanum* sequences are shown in Table 3.

For population ver03-392, 2 bands were successfully sequenced, but only band 392-9H1, see Table 3, gave high similarity scores, higher than 75%, with *Solanum* sequences from Genbank. Good hits were found with *S. lycopersicum* chromosome 11 clone C11HBa0119D16, complete sequence, and later with *S. lycopersicum* DNA, chromosome 8, clone: C08SLm0114A09, complete sequence. For population ver03-394, 5 different NBS bands could be sequenced successfully, 4 bands gave high similarity scores with *Solanum* sequences from Genbank. One of them (394_9H1) was identical to the 392-9H1 band. The bands 394_H1, 394_R1, and 394_R2 are identical in the overlapping region. The bands 394_H1 and 394_T1 show a 98% similarity in the overlapping region. The sequences

found in the NCBI database that showed similarity with the NBS markers of ver03-394 were identical to those found for population ver03-392.

For population snk7458, sequences of 5 NBS profiling bands could be successfully retrieved. Among each other the fragments show between 70 and 92% similarity in the overlapping regions. For all 5 bands, the highest similarity was found with *S. lycopersicum* BAC clone Clemson_Id 127E11. Park et al. (2005a) showed that this BAC clone contains several RGA sequences that are similar to the *Rpi-blb3* gene of *S. bulbocastanum* which is located on chromosome 4 of potato (Park et al. 2005a). Another high similarity score with bands from snk7458 was found with a *S. lycopersicum* DNA sequence from clone SL_MboI-40B16, also located on chromosome 4.

For population cap7358, the only band, cap7358_3M1, found in the NBS profiling analysis with the bulk and the individual samples was sequenced. After direct sequencing, it appeared that the band was a mixture between at least two fragments. The band was, therefore, cloned and sequenced. As much as 5 out of 10 cloned sequences were almost identical, 2 others were almost identical to each other but different from the others, and the last 3 were different from all. The sequence of the five identical fragments gave, when compared with the sequences in the Genbank database, an identity score of 91% with an E value of $7.00E-27$ (Table 3) with *S. tuberosum* mRNA for the NL27 protein. Hehl et al. (1999) located the gene encoding the NL27 protein on chromosome 11.

Confirmation of map position of genes

To verify the deduced map positions, we used markers that were expected to be (closely) linked based on their position on the potato maps (<http://www.gabipd.org/database/maps.html> and <http://www.sgn.cornell.edu/>). For each population, flanking markers were tested on the parents and all offspring that could be scored as clearly resistant or susceptible. The results are presented in Table 4. For populations ver03-392 and ver03-394 sequence similarity suggested that the resistance gene was positioned on chromosome 11. Several CAPS markers for chromosome 11 were tested, but none of those displayed any polymorphisms. The marker sequence was compared to a sequence database containing NBS profiling marker sequences that were mapped in the SHxRH potato mapping population (van der Linden et al., unpublished results), and was found to be nearly identical to a marker mapped on chromosome 6. This mapping position was confirmed by marker CD67 digested with enzyme HpyCH4IV and with enzyme *Ssi*I that both produced a polymorphic band that co-segregated with the resistance (see Fig. 2). An extra band is visible in the resistant parent

Table 3 Blast results of the NBS bands against the NCBI nucleotides database

Population	NBS band (genbank accession number)	Linkage arrangement	Length NBS band in bp	Accession from NCBI	Description given in NCBI	E value	Max indent (%)
ver03-392	392_9H1 (GU060647)	Coupling	382	AC171735.3	<i>Solanum lycopersicum</i> chromosome 11 clone C11HBa0119D16, complete sequence	1.00E-69	78
	392_9H1 (GU060647)	Coupling	382	AP010265.1	<i>Solanum lycopersicum</i> DNA, chromosome 8, clone: C08SLm0114A09, complete sequence	2.00E-68	78
	392_9H1 (GU060647)	Coupling	382	CU928132.3	<i>S. lycopersicum</i> DNA sequence from clone SL_MboI-49I21 on chromosome 4, complete sequence	2.00E-67	78
ver03-394	394_9H1 (GU060649)	Coupling	382	AC171735.3	<i>Solanum lycopersicum</i> chromosome 11 clone C11HBa0119D16, complete sequence	1.00E-69	78
	394_9H1 (GU060649)	Coupling	382	AP010265.1	<i>Solanum lycopersicum</i> DNA, chromosome 8, clone: C08SLm0114A09, complete sequence	2.00E-68	78
	394_9r1 (GU060651)	Coupling	284	AC171735.3	<i>Solanum lycopersicum</i> chromosome 11 clone C11HBa0119D16, complete sequence	6.00E-53	81
	394_9r1 (GU060651)	Coupling	284	AP010265.1	<i>Solanum lycopersicum</i> DNA, chromosome 8, clone: C08SLm0114A09, complete sequence	4.00E-49	80
	394_9R2 (GU060652)	Coupling	284	AC171735.3	<i>Solanum lycopersicum</i> chromosome 11 clone C11HBa0119D16, complete sequence	5.00E-54	81
	394_9R2 (GU060652)	Coupling	284	AC215440.2	<i>Solanum lycopersicum</i> chromosome 2 clone C02HBa0323C04, complete sequence	3.00E-50	80
	394_9T1 (GU060653)	Coupling	340	AC171735.3	<i>Solanum lycopersicum</i> chromosome 11 clone C11HBa0119D16, complete sequence	1.00E-49	75
	394_9T1 (GU060653)	Coupling	340	AP010265.1	<i>Solanum lycopersicum</i> DNA, chromosome 8, clone: C08SLm0114A09, complete sequence	6.00E-48	75
snk7458	7458_2A2 (GU060654)	Coupling	431	CU326358.5	<i>S. lycopersicum</i> DNA sequence from clone SL_MboI-40B16 on chromosome 4, complete sequence	1.00E-126	91
	7458_2A2 (GU060654)	Coupling	431	AF411807.1	<i>Lycopersicon esculentum</i> BAC clone Clemson_Id 127E11, complete sequence	1.00E-120	85
	7458_2M2 (GU060655)	Coupling	513	CU326358.5	<i>S. lycopersicum</i> DNA sequence from clone SL_MboI-40B16 on chromosome 4, complete sequence	2.00E-171	94
	7458_2M2 (GU060655)	Coupling	513	AF411807.1	<i>Lycopersicon esculentum</i> BAC clone Clemson_Id 127E11, complete sequence	2.00E-176	86
	7458_2M3 (GU060656)	Repulsion	431	CU326358.5	<i>S. lycopersicum</i> DNA sequence from clone SL_MboI-40B16 on chromosome 4, complete sequence	2.00E-131	94
	7458_2M3 (GU060656)	Repulsion	431	AF411807.1	<i>Lycopersicon esculentum</i> BAC clone Clemson_Id 127E11, complete sequence	7.00E-124	87

Table 3 continued

Population	NBS band (genbank accession number)	Linkage arrangement	Length NBS band in bp	Accession from NCBI	Description given in NCBI	E value	Max indent (%)
cap7358	7458_2M4 (GU060657)	Repulsion	238	AF411807.1	<i>Lycopersicon esculentum</i> BAC clone Clemson_Id 127E11, complete sequence	2.00E-79	94
	7458_2M4 (GU060657)	Repulsion	238	AF411807.1	<i>Lycopersicon esculentum</i> BAC clone Clemson_Id 127E11, complete sequence	2.00E-78	94
	7458_2M5 (GU060658)	Repulsion	203	CU326358.5	<i>S. lycopersicum</i> DNA sequence from clone SL_MboI-40B16 on chromosome 4, complete sequence	2.00E-37	86
	7458_2M5 (GU060658)	Repulsion	203	AF411807.1	<i>Lycopersicon esculentum</i> BAC clone Clemson_Id 127E11, complete sequence	5.00E-33	84
	7358_3M1 (1–5) (GU060659) (GU060660) (GU060661)	Repulsion	130	AJ009720.1	<i>Solanum tuberosum</i> mRNA for NL27 protein	7.00E-27	91

Only sequenced NBS bands that showed a similarity score of >75% with a *Solanum* sequence are shown. Also, only the best (small E value and a high maximum identity) hits to *Solanum* sequences in the NCBI database are shown

and the resistant offspring in both *S. verrucosum* populations.

The *P. infestans* resistance in population snk7458 was suggested to be located in the same cluster as the *Rpi-blb3* gene on chromosome 4. Several markers for chromosome 4, such as TG370, Th21, TG506R, CT229, and T1430, were tested. The parents often showed polymorphisms, but the offspring was almost always homogeneous for the same marker. Therefore, another approach was taken to find segregating markers. The PCR products of markers TG506, AF411807R, T1430, TG370, and Th21 from 6 resistant and 5 susceptible individuals were sequenced and checked for SNPs. The only polymorphism that was found between the resistant and susceptible individuals was in the PCR product of marker Th21. This SNP was shown to co-segregate with resistance. The SNP is located in an *MboI* site in the middle of the PCR fragment (TGATC for the susceptible, T[A/G]ATC for the (heterozygous) resistant individuals). A PCR with Th21 followed by digestion with *MboI* on all the available individuals, resulted in an extra band for the resistant parent *S. schenckii* GLKS 30659 and an extra band for 27 out of 39 resistance phenotypes. All the 5 susceptible phenotypes lacked the extra band. This implies that at least 12 out of 45 resistant plants contain a second gene conferring resistance to *P. infestans*.

To confirm the position of the marker in population cap7358, several SSR and CAPS primers for chromosome 11 were tested on the parents, the bulks and the individuals of this population. The CAPS marker CP58 in combination with restriction enzyme *MspI* produced an extra band in the resistant parent and in 18 out of 18 susceptible offspring, showing that the band was segregating in repulsion phase. Thirty one out of the 32 resistant individuals lacked this extra band.

Discussion

A new strategy for mapping resistance genes

In the present study we describe a novel approach to identify markers linked to resistance genes. The first step in the approach consists of producing small populations segregating for *P. infestans* resistance, phenotyping the populations for resistance, and composing bulks of resistant and susceptible individuals. Then, the bulks are genotyped using NBS profiling to obtain markers that co-segregate with resistance, followed by sequencing of co-segregating NBS fragments and BLAST analysis to identify the fragment. Combining this information with literature data on mapping of resistance genes results in a suggestion for a putative map position. Finally, the map positions are confirmed using known flanking markers.

Table 4 Confirmation of the putative map positions

Population	Locus	Chrom. Nr.	Forward and reverse primer ^a	Enzyme used	Confirmation with CAPS markers ^b
ver03-392	CD67	6	F: CCCCTGCAAATCCGTACATA R: CCATACGAGTTGAGGGATCG	HpyCH4IV, SsiI	5/5 R 3/3 S
ver03-394	CD67	6	As Ver03-392	As Ver03-392	6/7 R 5/5 S
snk7458	Th21	4	F: ATTCAAAATTCTAGTTCGGCC R: AACGGCAAAAAGCACCAC	MboI	27/39 R 5/5 S
cap7358	CP58	11	F: ATGTATGGTTCGGGATCTGG R: TTAGCACCAACAGCTCCTCT	MspI	31/32 R ^c 18/18 S

Confirmation with CAPS markers indicates how many of the phenotypically resistant or susceptible individuals were confirmed by the marker analysis

^a F: forward primer, R: reverse primer

^b R resistant individuals (confirmed/tested), S susceptible individuals (confirmed/tested)

^c For 2 from the 34 resistant individuals the PCR failed so no data on this marker could be retrieved

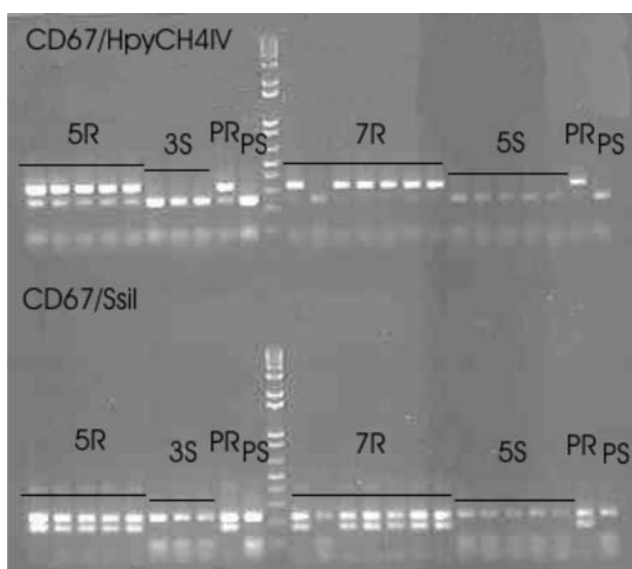


Fig. 2 Marker CD67 shows co-segregation with *P. infestans* resistance in populations ver03-392 and ver03-394 after digestion with HpyCH4IV and digestion with SsiI

Large differences were observed in the number of markers co-segregating with resistance in the 3 different populations, ranging from 1 in population cap7358 to 33 in the ver03-392 population (Table 2). These differences are possibly caused by the position of the targeted R gene. A high number of polymorphic bands co-segregating with resistance probably means that they are part of a large cluster of R genes. Few or no polymorphic NBS bands could mean that the parents were closely related and that the targeted R gene has an isolated position. In case no polymorphisms are detected with the 25 primer–enzyme combinations additional enzymes or primers may be tested. Furthermore, there is always a chance that the resistance gene under

study is not of the NBS-LRR type but belongs to another class of resistance genes (Ingvarsdson et al. 2008). The polymorphisms identified in the bulks that could not be confirmed in the analysis of the individual genotypes were due to differences in band intensity. The polymorphisms that showed a clear presence/absence of bands could be confirmed.

Many NBS profiling bands that were sequenced could be annotated successfully as being from a putative NBS-LRR type of resistance gene (results not shown). Correct annotation depends on the availability of sufficient sequence information in the databases. In addition, highly homologous sequences may sometimes be found in different clusters, as was shown for *Mi* and *I2* homologs (van der Vossen et al. 2005; Seah et al. 2007). This may complicate mapping afterwards, as was shown for fragment 392_9H1, obtained from population ver392. For this fragment, high similarity was found with a sequence of *S. lycopersicum* which had previously been mapped to chromosome 11 (Mueller et al. 2005, 2008), while another sequence homolog was retrieved from chromosome 4 (McGuire 2009). The putative positions on chromosome 11, or chromosomes 8 or 4, (Table 3), as suggested by BLAST with the NCBI database for several bands from the populations ver03-392 to ver03-394, could not be verified. The putative map position on chromosome 6 for this marker, inferred from the high sequence similarity to a mapped NBS profiling marker (van der Linden et al., unpublished results) could be verified. This indicates that the NCBI database is still far from complete. With the increasing amount of data in public sequence databases and with the progress of the potato genome sequencing (PGSC, <http://www.potatogenome.net>) it is likely that in the near future new fragments can be mapped more efficiently with higher accuracy.

Identification and mapping of *P. infestans* resistance genes in *S. verrucosum*, *S. schenckii*, and *S. capsicibaccatum*

In the ver03-392 and ver03-394 populations, the resistance against *P. infestans* is located on chromosome 6, near marker CD67. Population 03-394 showed one recombinant for the polymorphism found with marker CD67. We have named the gene underlying the resistance *Rpi-ver1*. As we could not find any other co-segregating markers for the resistance, it is not clear whether the gene is positioned downstream or upstream of marker CD67. The marker CD67 itself is positioned at 10.5 cM according to the potato map Potato-TXB 1992 v27 (<http://www.sgn.cornell.edu/>). Resistance against *P. infestans* in *S. verrucosum* has been reported by van Soest et al. (1984). Rivera-Peña and Molina-Galan (1989) and Rivera-Peña (1990) studied the occurrence of late blight on naturally occurring populations of wild *Solanum* species on the slope of Nevado de Toluca for many years. He found highly resistant natural populations of *S. verrucosum*. Furthermore, a *P. infestans* resistance screening of the Commonwealth potato collection also yielded a very resistant *S. verrucosum* accession (Bradshaw et al. 2006). Whether any of the genes involved is similar to the *Rpi-ver1* remains to be established. Finally, Liu and Halterman (2006) reported on *P. infestans* resistance in *S. verrucosum*. They have identified a gene sharing 83.5% nucleotide identity with *Rpi-blb1*. It will be interesting to see whether this gene maps to the same position on chromosome 8 as the original *Rpi-blb1* (van der Vossen et al. 2003) or to the same position as *Rpi-ver1* on chromosome 6, or to a complete new position.

There are no indications that the sequences of the NBS markers of populations ver03-392 and ver03-394 show any similarity to the original *Rpi-blb1*.

In the snk7458 population, there is probably more than 1 resistance gene against *P. infestans* segregating, since the offspring consists of far more resistant than susceptible phenotypes (39:5). Furthermore, only 2/3 of the resistant genotypes contained the linked CAPS marker. None of the susceptible phenotypes contained the CAPS marker. This result suggests the possible presence of another gene or QTL that confers the phenotypic resistance. This situation is very similar to that described by Wang et al. (2008a) for *S. stoloniferum*. Based on the results, one would also expect the presence of polymorphic NBS bands with <100% co-segregation. However, the five NBS markers tested on 6 resistance and 4 susceptible individuals from the bulk showed 100% co-segregation for the NBS markers. It is possible that the polymorphic NBS bands that were linked to the other gene were not discovered in the first round of NBS profiling on the bulked individuals. One gene conferring resistance in the *S. schenckii* population snk7458, and mapped in this study is most likely located on chromosome

4, near or on marker Th21 (Table 4). We call the gene underlying the resistance *Rpi-snk1*. Our results indicate that *Rpi-snk1* is a member of a cluster of *Rpi-blb3* homologs on chromosome 4 (Park et al. 2005b). It co-segregates with the Th21 marker, and therefore it is also similar to *Rpi-abpt*, *R2*, and *R2-like* that all reside in the same R gene cluster on chromosome 4 (Park et al. 2005b). According to the phenetic and phylogenetic results of Jacobs et al. (2008), *S. schenckii* is closely related to *S. hougasii*. Accessions from *S. hougasii* are reported to show high resistance against *P. infestans* (Bradshaw et al. 2006). It will be interesting to see whether these accessions also carry the same resistance genes as found in *S. schenckii*.

In population cap7358, a gene conferring resistance against *P. infestans* was found on chromosome 11, near marker CP58. The marker identified is in repulsion phase. In the 50 offsprings tested with the CP58 marker only one individual had lost linkage with this marker. The newly found gene is named *Rpi-cap1*. The position of marker CP58 is at the top of the chromosome 11, on 0.00 cM according to the data from the map Potato-TXB 1992 V27 (<http://www.sgn.cornell.edu/>).

Another resistance gene that was mapped to this region is *R-mc1* (mapped at 66 cM in the functional map of chromosome 11 of potato for pathogen resistance, as published at <http://www.gabipd.org/database/maps.shtml>), which is a resistance gene against root-knot nematode *Meloidogyne chitwoodi* retrieved from *S. bulbocastanum* (Brown et al. 1996). Note that the orientation of this potato map of chromosome 11 is reversed compared to the previously mentioned SGN potato map of chromosome 11. Resistance against *P. infestans* in *S. capsicibaccatum* was reported by van Soest et al. (1984) and Ruiz de Galarreta et al. (1998) but no further details on sequence or position of this R gene are available.

Very recently, Pel et al. (2009) described the cloning of two *P. infestans* resistance genes from *S. venturii*. The genes were mapped to chromosome 9 using NBS profiling. The NBS profiling fragments were sequenced and identified as a *Tm-2²* homologs. They designed degenerated primers based on all known *Tm-2²*, which enabled them to clone the genes involved. This example shows the versatility of NBS profiling in combination with an allele mining strategy for quickly identifying and cloning of resistance genes.

In the mapping populations used in this study no new resistance gene clusters in *Solanum* material were found. The *P. infestans* resistance was derived from different wild *Solanum* species in which previously no resistance genes had been identified (though some species had been reported to express some *P. infestans* resistance). Although we used these relatively unknown sources, it seems that the genes conferring the resistance are linked to known clusters of

resistance genes. This may suggest that the present view on the *Solanum* genome is rather exhaustive and that most resistance clusters are already known. In a previous study, new resistance genes in *Solanum* derived from wild *Solanum* species, could also be positioned at already known R gene clusters of the genome. Wang et al. (2008a) found that the dominant R genes *Rpi-sto1* (derived from *S. stoloniferum*) and *Rpi-plt1* (from *S. polytrichon*) resided at the same position on chromosome 8 as *Rpi-blb1* in *S. bulbocastanum*. Possibly the found R genes on known loci constitute new alleles but new alleles can be positively identified with the aid of effector proteins (Vleeshouwers et al. 2008).

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