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ORIGINAL PAPER

# Allele mining in *Solanum*: conserved homologues of *Rpi-blb1* are identified in *Solanum stoloniferum*

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**Abstract** Allele mining facilitates the discovery of novel resistance (R) genes that can be used in breeding programs and sheds light on the evolution of R genes. Here we focus on two R genes, *Rpi-blb1* and *Rpi-blb2*, originally derived from *Solanum bulbocastanum*. The *Rpi-blb1* gene is part of a cluster of four paralogues and is flanked by *RGA1-blb* and *RGA3-blb*. Highly conserved *RGA1-blb* homologues were discovered in all the tested tuber-bearing (TB) and non-tuber-bearing (NTB) *Solanum* species, suggesting *RGA1-blb* was present before the divergence of TB and NTB *Solanum* species. The frequency of the *RGA3-blb* gene was much lower. Interestingly, highly conserved *Rpi-blb1* homologues were discovered not only in *S. bulbocastanum* but also in *Solanum stoloniferum* that is part of the series *Longipedicellata*. Resistance assays and genetic analyses in several F1 populations derived from the relevant late blight resistant parental genotypes harbouring the conserved *Rpi-blb1* homologues, indicated the presence of four dominant R genes, designated as *Rpi-sto1*, *Rpi-plt1*, *Rpi-pta1* and *Rpi-pta2*. Furthermore, *Rpi-sto1* and *Rpi-plt1* resided at the same position on chromosome VIII as *Rpi-blb1* in *S. bulbocastanum*. Segregation data also indicated that an additional unknown late blight resistance gene was present in three populations. In contrast to *Rpi-blb1*, no homologues of *Rpi-blb2* were detected in any material examined. Hypotheses are proposed to explain the presence of conserved *Rpi-blb1* homologues in *S. stoloniferum*. The discovery of conserved homologues of *Rpi-blb1* in EBN 2 tetraploid species offers the possibility to more easily transfer the late blight resistance genes to potato varieties by classical breeding.

## Introduction

Late blight is caused by the oomycete *Phytophthora infestans* (Mont.) de Bary and it is one of the most important diseases affecting the potato crop *Solanum tuberosum* L. worldwide. The management of the disease has been estimated to cost $ 3.5 billion annually (GILB 2004). To reduce the cost of the disease and the environmental damage, it is important to identify resistance that can be used in breeding programs. In the past, 11 major resistance genes (R-genes) were introgressed from hexaploid *S. demissum* into cultivated potato (Black et al. 1953; Malcolmson and Black 1966) and all these genes confer race-specific resistance. Unfortunately, the resistance based on these genes

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was quickly overcome by the pathogen. Hence, new sources of resistance are required to develop late blight resistant potato varieties.

More late blight R-genes have been found and mapped in wild diploid species. Examples are *S. pinnatisectum* (Kuhl et al. 2001) and *S. bulbocastanum* (Naess et al. 2000; Song et al. 2003; Van der Vossen et al. 2003 and 2005; Park et al. 2005) from Mexico and *S. berthaultii* (Rauscher et al. 2006; Ewing et al. 2000), *S. microdontum* (Sandbrink et al. 2000), *S. mochiquense* (Smilde et al. 2005) and *S. paucissectum* (Villamon et al. 2005) from Andean countries. In addition, the cultivated diploid *S. phureja* has been described to contain valuable resistance (Ghislain et al. 2001; Sliwka et al. 2006). The presence of late blight R-genes in the above-mentioned species indicates that wild and so-called primitive germplasm is a rich source for novel R-genes that may be exploited in breeding programs.

To date, four late blight R-genes have been cloned: *R1* (Ballvora et al. 2002), *R3a* (Huang et al. 2005), *RB* or *Rpi-blb1* (Song et al. 2003; van der Vossen et al. 2003) and *Rpi-blb2* (van der Vossen et al. 2005). The latter two genes were cloned from *S. bulbocastanum* and confer resistance to all *P. infestans* isolates tested so far. *Rpi-blb1* is part of a resistance gene analog (RGA) cluster of four members *RGA1-blb, Rpi-blb1*, *RGA3-blb*, and *RGA4-blb* on chromosome VIII (van der Vossen et al. 2003). Complementation analysis showed that only the genetic construct harbouring *Rpi-blb1* was able to complement the susceptible phenotype. The sequence relationships between *Rpi-blb1*, *RGA1-blb* and *RGA3-blb* showed that the *Rpi-blb1* gene most likely evolved from intragenic recombination between the ancestral genes of *RGA3-blb* and *RGA1-blb* (van der Vossen et al. 2003). *Rpi-blb2* resides in a locus harbouring at least 15 *Mi* gene homologues on chromosome VI, and the *Rpi-blb2* protein shows 82% sequence identity to the *Mi-1* protein (van der Vossen et al. 2005). *Mi-1* is a gene of tomato that confers resistance to the root knot nematode *Meloidogyne incognita* (Milligan et al. 1998).

This study aims at analysing the allelic frequency and variation of *Rpi-blb1* and *Rpi-blb2* in a large number of tuber-bearing *Solanum* species and it explores the genomic organization of the *Rpi-blb1* cluster in these species. Insight into allelic diversity may facilitate discovery of functional homologues that can be exploited in breeding programs and may also help to understand the evolution of R-genes.

## Materials and methods

### Plant material and DNA extraction

Material representing most of the section *Petota*, based on their systematic relationships derived from AFLP and NBS profiling data was selected (Wang et al. submitted). Seeds were obtained from several genebanks (Table 1). Individual seeds were surface-sterilized and sown in vitro on MS medium supplemented with 20% sucrose (Murashige and Skoog 1962) at 18°C and allowed to germinate for at least 6 weeks to obtain individual clones. In total, 86 genotypes covering 47 species representing 13 series (three genotypes from series *Etuberosa*) were used (Table 1). DNA was extracted according to the method described by Stewart and Via (1993). Species names and abbreviations follow Hawkes (1990), since the genebanks label material as such. However, we refer to *S. stoloniferum* (sensu Spooner et al. 2004) to indicate their broader species concept, which considers *S. fendleri, S. papita* and *S. polytrichon* as synonyms of *S. stoloniferum.*

### R-gene specific primers

All *Rpi-blb1* primers designed in this study (Table 2) were tested on the clone 8005-8 (BGRC accession number 8005, individual plant 8) from which the *Rpi-blb1* gene and its paralogues *RGA1-blb* and *RGA3-blb* were cloned. *Rpi-blb2* primers (Table 2) were tested on the late blight resistant clone Blb2002, the diploid *S. bulbocastanum* clone from which *Rpi-blb2* was cloned (van der Vossen et al. 2005).

### Segregating populations and resistance assays

In the case of *S. stoloniferum*, a late blight resistant clone CGN17605-4 was crossed with the susceptible breeding line RH89-039-16, which produces 2*n* pollen and is frequently used for mapping research at the Laboratory of Plant breeding at Wageningen University and Research Centre (Rouppe van der Voort et al. 1998; Huang et al. 2004; Park et al. 2005). For the late blight resistance test, detached leaf assays (DLAs) were performed as described by Vleeshouwers et al. (1999). The *P. infestans* isolates IPO82001 (race structure: 1, 2, 3 ,4, 5, 6, 7, 10, 11) and IPO655-2A (race structure 1, 2, 3, 4, 5, 6, 7, 8, 10,11) (Flier et al. 2003) were from the collection of PRI, isolate "Marknesse" (race structure: 1, 2, 3, 4, 6, 7, 10, 11) was from a diseased potato collected in 2005 near the Dutch village Marknesse.

Three other populations were constructed using plant material that is now known as *S. stoloniferum* (sensu Spooner et al. 2004). However, in the genebank catalog these materials are still present under their previous names *S. papita* and *S. polytrichon*. Two tetraploid mapping populations for *S. papita* (Pta), Pta 04-323 and Pta 04-325, were obtained by backcrossing two resistant offspring, Pta 03-390-1 and Pta 03-390-3 respectively, with a susceptible *S. polytrichon* (Plt) pollen donor from accession CGN 17751. Pta 03-390-1 and Pta 03-390-3 were both derived from a

**Table 1** Materials used for identification of *Rpi-blb1* and *Rpi-blb2* homologues and amplification of *Rpi-blb1* cluster members with the primer pairs BLB1F/R, RGA1F/R and RGA3F/R

| Species[a] | Genebank[b] | BLB1F/R | RGA1F/R | RGA3F/R |
|---|---|---|---|---|
| *S. etuberosum* | 18242 | 0 | 1 | 0 |
| *S. fernandezianum* | 18360 | 0 | 1 | 0 |
| *S. palustre* | 18241 | 0 | 1 | 0 |
| *S. acaule* subsp. *acaule* | BGRC7949 | 0 | 1 | 0 |
| *S. acaule* subsp. *aemulans* | 21331 | 0 | 1 | 0 |
| *S. ajanhuiri* | 18239 | 0 | 1 | 1 |
| *S. berthaultii* | 20644, 20650 | 0 | 1 | 1 |
| *S. brachistotrichium* | 17681 | 0 | 1 | 0 |
| *S. brachycarpum* | 17721(3),18347, CPC7028, GLKS1686 | 0 | 1 | 0 |
| *S. brevicaule* | 18231 | 0 | 1 | 0 |
| *S. bukasovii* | 17824 | 0 | 1 | 0 |
| *S. bulbocastanum* | 17687 | 1 | 1 | 1 |
| *S. bulbocastanum* | 17691 | 0 | 1 | 1 |
| *S. canasense* | 17589 | 0 | 1 | 0 |
| *S. cardiophyllum* | 18326 | 0 | 1 | 1 |
| *S. chacoense* | 18248-1, 18248-4 | 0 | 1 | 1 |
| *S. chacoense* | 18248-9 | 0 | 1 | 0 |
| *S. circaeifolium* | 18133 | 0 | 1 | 0 |
| *S. circaeifolium* subsp. *quimense* | 18127 | 0 | 1 | 0 |
| *S. demissum* | 20571 | 0 | 1 | 0 |
| *S. fendleri* subsp. *arizonicum* | PI497996 | 0 | 1 | 0 |
| *S. guerreroense* | 18290(2), GLKS1512 | 0 | 1 | 0 |
| *S. hjertingii* | 18345 | 0 | 1 | 0 |
| *S. hondelmannii* | 18106, 18182(2) | 0 | 1 | 0 |
| *S. hougasii* | 18339(2) | 0 | 1 | 0 |
| *S. huancabambense* | 17719 | 0 | 1 | 0 |
| *S. iopetalum* | 20561 | 0 | 1 | 1 |
| *S. iopetalum* | 20562 | 0 | 1 | 0 |
| *S. jamesii* | 18349 | 0 | 1 | 0 |
| *S. leptophyes* | 18140 | 0 | 1 | 0 |
| *S. lesteri* | 18337 | 0 | 1 | 1 |
| *S. megistacrolobum* | GLKS5422 | 0 | 1 | 0 |
| *S. microdontum* | 17596 | 0 | 1 | 1 |
| *S. microdontum* subsp. *gigantophyllum* | 18046, 18200 | 0 | 1 | 0 |
| *S. mochiquense* | 18263(2) | 0 | 1 | 0 |
| *S. oxycarpum* | 20558 | 0 | 1 | 0 |
| *S. papita* | 17831 | 1 | 1 | 1 |
| *S. paucissectum* | PI590922 | 0 | 1 | 0 |
| *S. phureja* | 18301 | 0 | 1 | 0 |
| *S. pinnatisectum* | 17745(3), 23012 | 0 | 1 | 1 |
| *S. polyadenium* | 17749 | 0 | 1 | 1 |
| *S. polytrichon* | 22361 | 0 | 1 | 0 |
| *S. raphanifolium* | 17753(2) | 0 | 1 | 0 |
| *S. sanctae-rosae* | 17837, 20576 | 0 | 1 | 0 |
| *S. schenckii* | 18361 | 0 | 1 | 1 |
| *S. sparsipilum* | 18221 | 0 | 1 | 0 |
| *S. sparsipilum* | 18225 | 0 | 1 | 1 |

**Table 1** continued

| Species[a] | Genebank[b] | BLB1F/R | RGA1F/R | RGA3F/R |
|---|---|---|---|---|
| *S. stoloniferum* | 17605, 17606, BGRC60465, CPC28 | 1 | 1 | 1 |
| *S. stoloniferum* | 18333, GLKS592 | 0 | 1 | 1 |
| *S. stoloniferum* | 17607, 18332, 18334, 18348, 23072, CPC12, GLKS512 | 0 | 1 | 0 |
| *S. sucrense* | 18205 | 0 | 1 | 0 |
| *S. tarijense* | 17861 | 0 | 1 | 1 |
| *S. tuberosum* subsp. *andigena* | 20614 | 0 | 1 | 1 |
| *S. vernei* | 21350 | 0 | 1 | 1 |
| *S. verrucosum* | 20567(2) | 0 | 1 | 0 |

[a] Materials are ordered alphabetically with the exception that three genotypes from series *Etuberosa* (non-tuber-bearing *Solanum* species) are listed in the front

[b] Materials starting with a number directly are from Center for Genetic resources, The Netherlands (CGN). Materials starting with BGRC, CPC, GLKS and PI are from Braunschweig Genetic Resources Collection (Germany), the Commonwealth Potato Collection (Dundee, Scotland), Gross Lusewitz (Germany), Potato Introduction Station, NRSP-6, Sturgeon Bay, Wisconsin (USA), respectively. Numbers in parentheses refer to the number of the genotype within one accession. Genotype number is provided only when PCR patterns within/among the genotypes differ. When more than one accession number is behind a particular species name, it indicates the representatives tested of these accessions show the same amplification profile with the three primer pairs used

**Table 2** Primers overview

| Primer | F/R | Sequence (5′–3′)[a] | Annealing temperature (°C) | Length of 72°C extension | Reference |
|---|---|---|---|---|---|
| BLB1F/R | F | AACCTGTATGGCAGTGGCATG | 58 | 50 s | |
| | R | GTCAGAAAAGGGCACTCGTG | | | |
| 517/1519 | F | CATTCCAACTAGCCATCTTGG | 58 | 50 s | |
| | R | TATTCAGATCGAAAGTACAACG | | | |
| 1521/518 | F | GAAAGTCTAGAGTTACACTGG | 58 | 50 s | |
| | R | CAATCACAATGGCAGGAACC | | | |
| BLB1F/R1 | F | AACCTGTATGGCAGTGGCATG | 55 | 1 min 40 s | |
| | R | GYTGTTAGGTGCTGCAATCC | | | |
| BLB1F1/R | F | GWGMATGGGAACATGTGAGAG | 55 | 1 min 40 s | |
| | R | GTCAGAAAAGGGCACTCGTG | | | |
| RGA1F/R | F | CAGTCACTTTCTTGTTTGCCG | 55 | 55 s | |
| | F | CAGTAGTGAAGTCACTGTGTG | | | |
| RGA3F/R | F | CATGCCTTAAGTCTCTAAGTTG | 55 | 55 s | |
| | R | TGGGAGTGAAGTAGCTTCTAC | | | |
| BLB2F/R | F | GGACTGGGTAACGACAATCC | 58 | 50 s | |
| | R | GCATTAGGGGAACTCGTGCT | | | |
| BLB2F/R1 | F | GGACTGGGTAACGACAATCC | 55 | 1 min 40 s | |
| | R | ATTTATGGCTGCAGAGGACC | | | |
| BLB2F1/R | F | ATTGCTGGARTCATTGCTGG | 55 | 1 min 40 s | |
| | R | GCATTAGGGGAACTCGTGCT | | | |
| 1 + 1′ | F | CACGAGTGCCCTTTTCTGAC | 50 | 2 min | Colton et al. (2006) |
| | R | ACAATTGAATTTTTAGACTT | | | |
| CT88 | F | GGCAGAAGAGCTAGGAAGAG | 60 | 50 s | van der Vossen et al. (2003) |
| | R | ATGGCGTGATACAATCCGAG | | | |

*F* forward primer; *R* reverse primer

[a] Nucleotide abbreviations according to the IUB code (M, AC; R, AG; W, AT; Y, CT)

cross between a susceptible plant from Pta accession CGN 18319 and a resistant plant from Pta accession CGN 17831. In 2004, 48 plants from this cross, which included Pta 03-390-1 and Pta 03-390-3, were found to be completely resistant in a single DLA with *P. infestans* isolate IPO82001. Similarly, a segregating population of Plt was obtained (Plt 04-281) by crossing resistant clone Plt 03-369-1 with a susceptible clone from Pta accession CGN18319. Plt 03-369-1 was derived from a cross between a susceptible plant from Pta accession CGN 18319 and a resistant plant from Plt accession CGN 18318. Again, this cross showed a fully resistant offspring after screening 72 plants in a DLA with the IPO82001 isolate. All *S. stoloniferum* accessions, including *S. papita* and *S. polytrichon* are EBN 2 tetraploids that display disomic inheritance (Hawkes 1990).

### PCR amplification

Three pairs of primers (BLB1F/R, 517/1519, 1521/518, Table 2) were designed based on the *Rpi-blb1* homologous sequences (van der Vossen et al. 2003), aiming at specific amplification of the *Rpi-blb1* gene. Upstream and downstream of the primers *BLB1F/R* new primers were designed to determine whether non-amplification with the BLB1F/R primer pair might be caused by a mutation in either primer site. In addition, primers for the *Rpi-blb1* paralogues *RGA1-blb* and *RGA3-blb* were developed. A similar approach was followed to design *Rpi-blb2* primers (Table 2). Figure 1 shows the locations for the *Rpi-blb1* and *Rpi-blb2* primers designed in this study. PCR reactions were carried out in a 15-μl reaction system, containing ~100 ng DNA, 2.25 pmol of each primer, 3 mM of each dNTP, 0.6 units Taq-poplymerase (15 U $\mu l^{-1}$, SphaeroQ, Leiden, The Netherlands), 10 mM Tris–HCl (pH 9), 1.5 mM $MgCl_2$, 50 mM KCl, 0.1% Triton X-100 and 0.01% (w/v) gelatine. The PCR protocol started with 5 min at 95°C. The 35-cycle amplification profiles were as followed: 30-sec DNA denaturation at 94°C, 40-sec annealing and variable elongation (depending on the primer, Table 2) at 72°C. The PCR was finalized by an extra 5-min elongation step at 72°C. The PCR protocol used for primer 1 + 1' was according to Colton et al. (2006).

The chromosome VIII specific marker CT88 (van der Vossen et al. 2003) was initially tested on the parents of all four segregation populations. Following digestion of the PCR products with specific restriction enzymes, polymorphic markers were subsequently tested in the entire population. All amplification reactions were performed in a Biometra® T-Gradient or Biometra® Uno-II thermocycler (Westburg, Leusden, the Netherlands). PCR products were separated in 1.5% agarose gel and stained with ethidiumbromide.

### Data collection and sequence analysis

For selected genotypes, PCR products were sequenced directly to confirm their identity and to identify single nucleotide polymorphisms (SNPs). Each fragment was sequenced from both sides using the two primers as a sequencing primer with the BigDye Terminatior kit. Sequencing reactions were analysed using an ABI 3700 automated sequencer (Applied Biosystems, USA). DNA sequences were analysed using DNAstar (Lasergene, Madison, WI, USA).

## Results

### Primer specificity

All *Rpi-blb1* and *Rpi-blb2* related primers designed in this study (Table 2) were tested on the clone BGRC accession

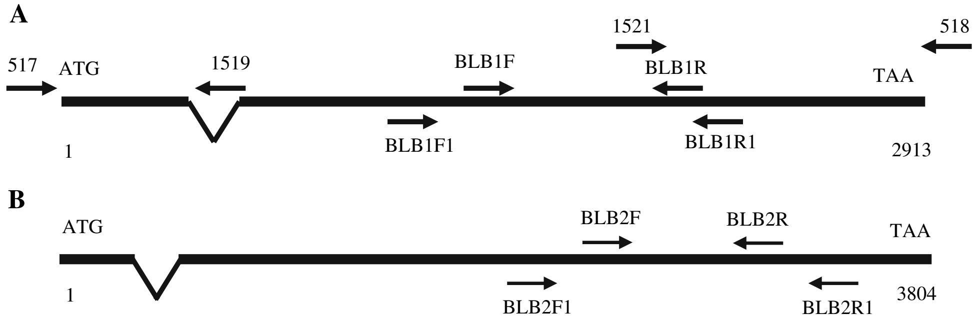

**Fig. 1** Location of the primers designed in this study, drawing not to scale. *Horizontal lines* indicate exons. *Lines angled downwards* indicate the position of the intron sequence. **a** *Rpi-blb1* related primers. Forward primer 517 from the primerpair 517/1519 is located upstream of the start codon of *Rpi-blb1*, while reverse primer 518 from the primerpair 1521/518 is located downstream of the stop codon. Primers BLB1F1 and BLB1R1 are located upstream and downstream of the primer BLB1F/R. Reverse primer 1519 is located in the intron of *Rpi-blb1*, while all the rest primers (except 517 and 1521) are located in the exon of *Rpi-blb1*. **b** *Rpi-blb2* related primers

8005 (individual 8005-8), from which *Rpi-blb1* and its paralogues *RGA1-blb* and *RGA3-blb* were cloned, or on clone Blb2002, which was used for the cloning of *Rpi-blb2* (van der Vossen et al. 2005). Sequence analysis showed that all primers amplified the expected fragments with two exceptions: (1) poor sequences for the BLB1F1/R primer product were obtained, suggesting that this primer pair amplified a mixture of related sequences; (2) compared with AY426261 (*RGA3-blb*), double peaks were found at five nucleotide positions. Except for these five SNPs, the remainder of the sequence was identical to AY426261.

### *RGA1-blb* and *RGA3-blb* homologues

With the RGA1F/R-specific primers, *RGA1-blb* homologues were amplified not only from all the tuber-bearing *Solanum* genotypes but also from three non-tuber-bearing *Solanum* genotypes *S. etuberosum, S. fernandezianum* and *S. palustre* (Table 1). RGA1F/R derived sequences from 36 randomly selected genotypes were highly homologous (96–99%) to that of *RGA1-blb* (results not shown). In contrast, a much smaller set of genotypes contained RGA3 homologues (Table 1). RGA3F/R derived sequences from different genotypes were highly homologous to *RGA3-blb* AY426261(88–98%).

### *Rpi-blb1* in *Solanum* species

Screening of more than 80 genotypes (Table 1) with three pairs of primers, primer pair BLB1F/R, BLB1F/R1 and BLB1F1/R (Table 2), showed that the primer BLB1F/R amplified fragments from genotypes in *S. bulbocastanum* and *S. stoloniferum* (sensu Spooner et al. 2004). In these genotypes, primer pairs BLB1F/R1 and BLB1F1/R also amplified fragments. Combined sequences from BLB1F/R and BLB1F/R1-derived fragments showed that these fragments were highly homologous to that of *Rpi-blb1*. BLB1F1/R amplicons from some genotypes contained the 18bp-sequence that is characteristic for the *Rpi-blb1* resistance allele (Song et al. 2003), while others did not. This indicated that the BLB1F1/R primer amplified both the R and S alleles. In some BLB1F1/R products heterogeneity was observed, indicating that more than one homologue was amplified. All genotypes that contained the *Rpi-blb1* specific allele also contained conserved *RGA1* and *RGA3* homologues.

In some genotypes, primer set BLB1F/R did not amplify fragments, while the primer set BLB1F/R1 did. These genotypes can be classified into three groups: genotypes showing (1) one single fragment of the expected size, (2) one single fragment of smaller size (data not shown) and (3) one single fragment of the expected size and the other of smaller size (data not shown). Sequences from the first group did not contain the reverse primer BLB1R, which explains why BLB1F/R primers did not amplify fragments. For this reason, these genotypes were excluded in further analyses.

After identifying the *Rpi-blb1* homologues, more accessions and genotypes (Table 3) of the series *Longipedicellata* (Hawkes 1990) were screened for the presence of *Rpi-blb1* homologues with the primers BLB1F/R, 517/1519 and 1521/518. As expected, more accessions and genotypes were found to contain highly conserved *Rpi-blb1* homologues (Table 3), which also harboured the 18-bpsequence that is characteristic for the functional *Rpi-blb1* gene (Song et al. 2003). Based on the obtained partial sequences, three haplotypes were discovered (Table 4). For haplotypes 1 and 3, SNPs at positions 64 and 65 together changed the amino acid from Val to Thr, while in haplotype 2, Val was changed to Ala. Another four SNPs at position 2664, 3134, 3255 and 3588 changed the amino acid from Lys to Arg, Met to Leu, Ala to Glu and Ile to Asn, respectively.

### Identification and mapping of the genes *Rpi-sto1, Rpi-plt1, Rpi-pta1 and Rpi-pta2*

Progenies (33 individuals) of a cross between *S. stoloniferum* 17605-4 that contained the conserved *Rpi-blb1* homologue and the breeding clone RH89-039-16 were evaluated for late blight resistance. This test resulted in 19 resistant and 14 susceptible genotypes, indicating that a single dominant R gene segregated in the population, which was designated as *Rpi-sto1*. Four primer pairs for *Rpi-blb1* (BLB1F/R, 517/1519, 1521/518 and 1 + 1') were tested on all the individuals of the population. All amplified fragments of the expected size co-segregated with the resistance. Figure 2 illustrates co-segregation between the BLB1F/R primer and the resistance. Subsequent digestion of amplicons of marker CT88 with the restriction enzyme *Hinf*I showed that the CT88 cosegregated with *Rpi-sto1* in repulsion phase (Fig. 3), suggesting that *Rpi-sto1* is located on the chromosome VIII at a position similar as the *Rpi-blb1* in *S. bulbocastanum* (van der Vossen et al. 2003).

Forty offspring clones of each of the populations Pta 04-323, Pta 04-325 and Plt 04-281 were tested in triplicate in two independent DLAs with *P. infestans* isolates 655-2A and "Marknesse". All three populations segregated resistance to late blight in these assays and for 36, 39 and 37 individuals, respectively, a clear phenotype could be determined. The percentages of resistant offspring in the groups were 66, 69 and 73%, respectively. For all three populations, the *Rpi-blb1* primer pairs BLB1F/R, 517/1519, 1521/518 and 1 + 1'(Table 2) produced positive results for some resistance genotypes, while other resistance genotypes produced negative results. None of the susceptible genotypes was positive for any of the *Rpi-blb1* related primers. The

**Table 3** Extended materials used to identify the *Rpi-blb1* and *Rpi-blb2* homologues

| Species | Genebank[a] | Primers for *Rpi-blb1* | | |
|---|---|---|---|---|
| | | BLB1F/R | 517/1519 | 1521/518 |
| *S. papita* | 17830(3), 18309(2), 18319(2) | 0 | 0 | 0 |
| *S. papita* | 17831(4) | 1 | 1 | 1 |
| *S. papita* | 17832-1, 17832-5 | 0 | 0 | 0 |
| *S. papita* | 17832-2 | 1 | 1 | 1 |
| *S. polytrichon* | 18318-(1 to 4), 18318-(6 to 9) | 0 | 0 | 0 |
| *S. polytrichon* | 18318-5 | 1 | 1 | 1 |
| *S. stoloniferum* | 17606, 17607, 18332, 18333, 18348(2), CPC12, GLKS512 | 0 | 0 | 0 |
| *S. stoloniferum* | 18334-1 | 0 | 0 | 0 |
| *S. stoloniferum* | 18334-8 | 0 | 0 | 1 |
| *S. stoloniferum* | 17605(4), BGRC60465-3 | 1 | 1 | 1 |

[a] Materials starting with a number directly are from Center for Genetic resources, The Netherlands (CGN). Materials starting with BGRC, CPC and GLKS are from Braunschweig Genetic Resources Collection (Germany), the Commonwealth Potato Collection (Dundee, Scotland), Gross Lusewitz (Germany), respectively. Numbers in parenthesis refer to the number of the genotypes included in that one accession. Genotype number is provided only when PCR patterns within/among the genotypes differ

**Table 4** Nucleotide polymorphisms of *Rpi-blb1* homologues from three pairs of primers BLB1F/R, 517/1519 and 1521/518. Rpi-blb1 sequences are as reference for comparison

| Species | Materials[b] | Sequences amplified from three pairs of primers[a] | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 28 | **64** | **65** | 123 | 210 | 315 | **2664** | **3134** | **3255** | **3588** | Number of SNP | Haplotype |
| | Rpi-blb1 | C | G | T | T | T | C | A | A | C | T | | |
| *S. papita* | 17831-1 | T | A | C | C | C | | G | C | | A | 8 | 1 |
| *S.. papita* | PTA 03-390-1 | T | A | C | C | C | | G | C | | A | 8 | 1 |
| *S. papita* | PTA 03-390-3 | T | A | C | C | C | | G | C | | A | 8 | 1 |
| *S. papita* | 17831-8 | T | A | C | C | C | | G | C | | A | 8 | 1 |
| *S. papita* | 17832-2 | T | A | C | C | C | | G | C | | A | 8 | 1 |
| *S. polytrichon* | PLT 03-369-1 | T | A | C | C | C | | G | C | | A | 8 | 1 |
| *S. stoloniferum* | BGRC60465-3 | | | C | | | T | | | A | | 3 | 2 |
| *S. stoloniferum* | 17605-1 | T | A | C | C | C | | | | | A | 6 | 3 |
| *S. stoloniferum* | 17605-2 | T | A | C | C | C | | | | | A | 6 | 3 |
| *S. stoloniferum* | 17605-3 | T | A | C | C | C | | | | | A | 6 | 3 |
| *S. stoloniferum* | 17605-4 | T | A | C | C | C | | | | | A | 6 | 3 |

Bold positions mean that the SNP changes the amino acid. Nucleotides are numbered only when SNP is available

[a] Sequences from start codon till position 542 are from the primer 517/1519 and sequences from 1737 till stop codon are from the combination of the primer BLB1F/R and 1521/518

[b] Materials starting with a number directly are from Center for Genetic resources, The Netherlands (CGN). Materials starting with BGRC are from Braunschweig Genetic Resources Collection (Germany). Genotype information is given after the genebank number. For details on the three genotypes of the segregating populations PTA 03-390-1, PTA 03-390-3, PLT 03-369-1, see Materials and methods

fact that all three populations consisted of about 75% resistant offspring and the fact that about one third of the resistant plants does not amplify a fragment with the specific primers, strongly suggests that there are two different R-genes segregating. One of the two genes is highly homologous to *Rpi-blb1* and the other one an unknown late blight R gene. The genes homologous to *Rpi-bb1l* were designated as *Rpi-plt1*, *Rpi-pta1* and *Rpi-pta2*. *HinfI* and *FspBI* digestion of CT88 PCR amplicons in population Plt 04-281 showed this marker to be genetically linked in coupling phase with resistance (results not shown), confirming that *Rpi-plt1* is also located on chromosome VIII.

## *Rpi-blb2* in *Solanum* species

*Rpi-blb2* homologues were not detected using the primer pair BLB2F/R in any of the wild species clones evaluated (Tables 1, 3), but the positive control from which the gene

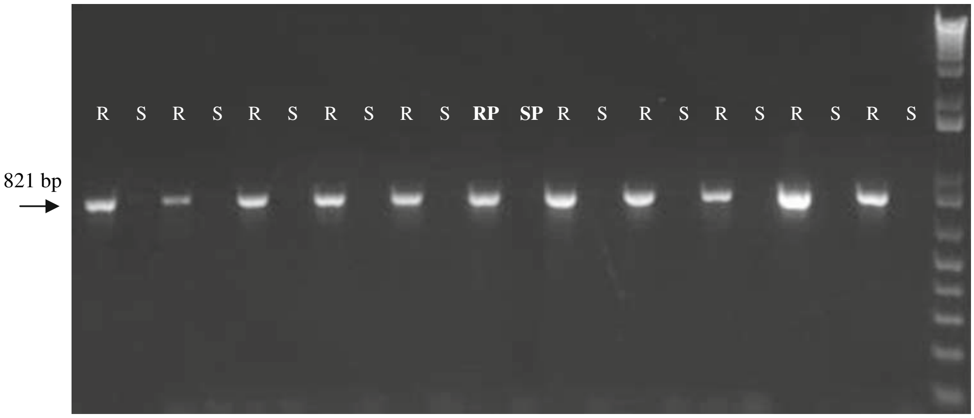

**Fig. 2** Co-segregation of resistance with BLB1F/R marker. PCR product (821 bp) was obtained with the BLB1F/R primerpair and is indicated by the arrow. R, S, RP and SP indicate the resistant offspring, susceptible offspring, resistant parent *S. stoloniferum* CGN17605 genotype 4, susceptible parent RH89-039-16, respectively. A marker-size ladder is indicated (1 kb+) in the last lane

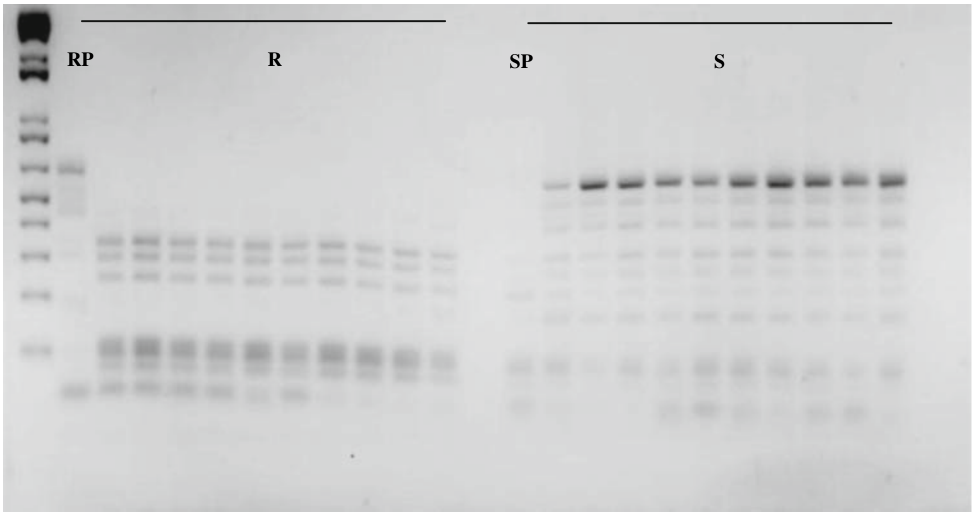

**Fig. 3** Linkage of *Rpi-sto1* to chromosome VIII marker CT88 in the *S. stoloniferum* population CGN17605-4xRH89-039-16. The polymorphism is revealed by *HinfI* digestion of the amplification product obtained with the CT88 marker. A marker-size ladder is indicated (1 kb+) in the first lane. RP means resistant parent *S. stoloniferum* CGN17605 genotype 4, followed by ten resistant offspring. SP represents the susceptible parent RH 89-039-16, followed by ten susceptible offspring

was cloned was indeed found to contain *Rpi-blb2*. For some genotypes, the other two primer pairs (BLB2F1/R and BLB2F/R1) amplified fragments, while for some other genotypes, only one of the two primer pairs amplified fragments. Sequence analysis from selected fragments showed that they had a low degree of homology to the sequence of the *Rpi-blb2* gene (not shown).

## Discussion

### Genomic organization of the *Rpi-blb1* gene cluster

Many R genes are present within clusters of tightly linked genes (Michelmore and Meyers 1998; Meyers et al. 2003). This is also the case for the *Rpi-blb1* gene (van der Vossen et al. 2003). Investigation of the presence or absence of the three paralogues *Rpi-blb1*, *RGA1-blb* and *RGA3-blb* showed that *RGA1-blb* homologues are present not only in all the tuber-bearing *Solanum* genotypes but also in non-tuber-bearing species (Table 1), suggesting that *RGA1-blb* was present before the divergence of tuber-bearing and non-tuber-bearing *Solanum* species. All 56 *RGA1-blb* fragments sequenced were highly homologous (96–99% sequence identity with *RGA1-blb*), indicating that *RGA1-blb* is well conserved. As *RGA1-blb* is likely to be expressed (van der Vossen et al. 2003), its presence in such a wide variety of species suggests that it might be a functional R gene and that it may confer resistance to a very common pathogen. Interestingly, whenever the conserved *Rpi-blb1* gene fragments were present in certain genotypes, both *RGA1-blb* and *RGA3-blb* homologues were also present (Table 1). On the contrary, genotypes that contained both *RGA1-blb* and *RGA3-blb* homologues did not necessarily contain the conserved *Rpi-blb1* gene homologue. *S. berthaultii* CGN 20644 and *S. chacoense* CGN18248 are two examples of accessions that contain homologues of both *RGA1-blb* and *RGA3-blb*, but not of *Rpi-blb1*, as judged from the amplification results with *Rpi-blb1* primers. The presence of *RGA1-blb* and *RGA3-blb* homologues (or their ancestors) seems to be essential for the generation of the *Rpi-blb1* gene homologue, as was suggested previously (van der Vossen et al. 2003).

### Allele mining for homologues of *Rpi-blb1* and *Rpi-blb2*

Four species (*S. stoloniferum*, *S. papita*, *S. polytrichon, S. fendleri*) in the series *Longipedicellata* as recognized by Hawkes (1990) have been considered conspecific (Spooner et al. 2004), as both morphological characteristics (Spooner et al. 2001) and molecular data (van den Berg et al. 2002) failed to separate them. In this study conserved *Rpi-blb1* homologues were found in *S. bulbocastanum* and *S. stoloniferum* (sensu Spooner et al. 2004). In the case of *S. stoloniferum* and *S. polytrichon* these *Rpi-blb1* homologues were confirmed to be linked to resistance and to the chromosome VIII marker CT88, which was previously shown to be linked to *Rpi-blb1* in *S. bulbocastanum*. All these data strongly suggest that *Rpi-sto1, Rpi-plt1, Rpi-pta1 and Rpi-pta2* are functional homologues of *Rpi-blb1*, and give additional support for the synonymy of *S. papita* and *S. polytrichon* to *S. stoloniferum*..

In contrast to *Rpi-blb1*, no *Rpi-blb2* homologues were discovered in any of the genotypes evaluated, indicating that *Rpi-blb2* is probably a gene that has evolved relatively recently. However, it may be possible to find more *Rpi-blb1* and *Rpi-blb2* alleles as our screening was not exhaustive.

### Origin of *Rpi-sto1, Rpi-plt1, Rpi-pta1* and *Rpi-pta2*

The sequentially and positionally conserved *Rpi-blb1* homologues were found in the advanced polyploid Central American species *S. stoloniferum* (sensu Spooner et al. 2004) from series *Longipedicellata*, which is considered distinct from the primitive diploid species *S. bulbocastanum* from series *Bulbocastana* (Hawkes 1990; Spooner et al. 2004). Thus, the question arises how the genes *Rpi-sto1, Rpi-plt1, Rpi-pta1 and Rpi-pta2* ended up in *S. stoloniferum* (sensu Spooner et al. 2004). The Central American polyploid species from series *Longipedicellata* are thought to have evolved from amphidiploidisations of a primitive Mexican ancestor with more advanced South American species (Hosaka et al. 1984; Hawkes 1990; Matsubayashi 1991). We propose two hypotheses. (1) *Rpi-blb1* is genetically highly conserved and was present in the wild ancestors of *S. stoloniferum* (sensu Spooner et al. 2004). Our data strongly suggest that *S. bulbocastanum* is one of the progenitors of *S. stoloniferum*. This hypothesis is supported by the similar constitution of the *Rpi-blb1* gene cluster (*RGA1-blb* and *RGA3-blb*) and the highly homologous sequences in *S. bulbocastanum* and *S. stoloniferum* (sensu Spooner et al. 2004) (Table 4). Furthermore, *Rpi-sto1* and *Rpi-plt1* were mapped in *S. stoloniferum* and *S. polytrichon* to the same chromosomal region of chromosome VIII as *Rpi-blb1* in *S. bulbocastanum*. (2) Alternatively, the *Rpi-blb1* homologues in *S. stoloniferum* (sensu Spooner et al. 2004) are the result of independent recombination events. However, the high level of sequence conservation of the *Rpi-blb1* homologues and previous cytogenetic studies (Hawkes 1990; Matsubayashi 1991) suggest the first alternative as being more likely.

Interestingly, the resistance to root-knot nematodes *Meloidogyne chitwoodi* and *M. fallax* is also found to be present in both *S. bulbocastanum* and *S. stoloniferum* (Janssen et al. 1995), indicating that other R genes in *S. stoloniferum* and *S. bulbocastanum* might share common ancestry, too.

### Potato late blight resistance breeding prospects

The *Rpi-blb1* gene was originally discovered and cloned from *S. bulbocastanum* (Song et al. 2003; van der Vossen et al. 2003, 2005), a species that cannot be crossed with the cultivated potato *S. tuberosum* directly. Our study suggests that putatively functional *Rpi-blb1* homologues are also present in *S. stoloniferum* (sensu Spooner et al. 2004), a species that can be crossed with cultivated potato directly (Jackson and Hanneman Jr 1999), although the crossing efficiency is low. Assuming that the *Rpi-sto1* gene has the same specificity as *Rpi-blb1* it may now be easier to introduce the *Rpi-blb1* resistance specificity into cultivated potato from *S. stoloniferum* (sensu Spooner et al. 2004) instead of *S. bulbocastanum*. This is supported by the fact that *S. stoloniferum* (CPC 2093) has been used to breed potato varieties ‘Kuras’, ‘Sante’, ‘Xantia’ and ‘Lady Christl’ (Hutten and van Berloo 2001). In contrast, making use of *S. bulbocastanum* is only possible through a tedious and time-consuming breeding scheme, for example, through bridge crosses (Hermsen and Ramanna 1973) or through somatic hybridisation (Helgeson et al. 1998).

We anticipate that for other resistance genes present in primitive species, a similar situation may exist, e.g. homologues being present in more advanced species that are more easily crossable with the cultivated potato. Therefore, before starting a breeding program with a species that cannot be crossed with cultivated potato directly, evaluation of directly crossable germplasm for the presence of that gene may speed up the breeding program and save time and money.

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## References

Ballvora A, Ercolano MR, Weiss J, Meksem K, Bormann CA, Oberhagemann P, Salamini F, Gebhardt C (2002) The R1 gene for potato resistance to late blight (*Phytophthora infestans*) belongs to the leucine zipper/NBS/LRR class of plant resistance genes. Plant J 30:361–371

Black W, Mastenbroek C, Mills WR, Peterson LC (1953) A proposal for an international nomenclature of races of *Phytophthora infestans* and of genes controlling immunity in *Solanum demissum* derivatives. Euphytica 2:173–179

Colton LM, Groza HI, Wielgus SM, Jiang J (2006) Marker-assisted selection for the broad-spectrum potato late blight resistance conferred by gene RB derived from a wild potato species. Crop Sci 46:589–594

Ewing EE, Simko I, Smart CD, Bonierbale MW, Izubuti ESG, May GD, Fry WE (2000) Genetic mapping from field tests of qualitative and quantitative resistance to *Phytophthora infestans* in a population derived from *Solanum tuberosum* and *Solanum berthaultii*. Mol Breed 6:25–36

Flier WG van den Bosch GBM, Turkensteen LJ (2003) Stability of partial resistance in potato cultivars exposed to aggressive strains of *Phytophthora infestans*. Plant Path 52:326–337

Ghislain M, Trognitz B, Herrera M, Solis J, Casallo G, Vasquez C, Hurtado O, Castillo R, Portal L, Orrillo M (2001) Genetic loci associated with field resistance to late blight in offspring of *S. phureja and S. tuberosum* grown under short-day conditions. Theor Appl Genet 103:433–442

GILB (2004) Global initiative on late blight, http://www.cipotato.org/gilb/

Hawkes JG (1990) The Potato-Evolution, biodiversity and genetic resources, Belhaven Press, London, UK

Helgeson JP, Pohlman JD, Austin S, Haberlach GT, Wielgus SM, Ronis D, Zambolim L, Tooley P, McGrath JM, James RV, Stevenson WR (1998) Somatic hybrids between *Solanum bulbocastanum* and potato: a new source of resistance to late blight. Theor Appl Genet 96:738–742

Hermsen JGTh, Ramanna MS (1973) Double-bridge hybrids of *Solanum bulbocastanum* and cultivars of *Solanum tuberosum*. Euphytica 22:457–466

Hosaka K, Ogihara Y, Matsubayashi M, Tsunewaki K (1984) Phlogenetic relationship between the tuberous *Solanum* species as revealed by restriction endonuclease analysis of chloroplast DNA. Jpn J Genet 59:349–369

Huang S, Vleeshouwers VGAA, Werij JS, Hutten RCB, van Eck HJ, Visser RGF, Jacobsen E (2004) The R3 resistance to *Phytophthora infestans* in potato is conferred by two closely linked R genes with distinct specificities. Mol Plant Microbe Interact 17:428–435

Huang S, van der Vossen E, Kuang H, Vleeshouwers VGAA, Zhang N, Borm TJA., van Eck HJ, Baker B, Jacobsen E, Visser RGF (2005) Comparative genomics enabled the isolation of the R3a late blight resistance gene in potato. Plant J 42:251–261

Hutten RCB, van Berloo R (2001) An online potato pedigree database. URL: http://www.dpw.wau.nl/pv/query.asp

Jackson SA, Hanneman RE Jr (1999) Crossability between cultivated and wild tuber-and non-tuber-bearing *Solanums*. Euphytica 109:51–67

Janssen GJW, van Norel A, Verkerk-Bakker B, Janssen R (1995) Resistance to *Meloidogyne chitwoodi*, *M. fallax* and *M. hapla* in wild tuber-bearing Solanum spp. Euphytica 92:287–294

Kuhl JC, Hanneman RE, Havey MJ (2001) Characterization and mapping of Rpi1, a late-blight resistance locus from diploid (1EBN) Mexican *Solanum pinnatisectum*. Mol Genet Genomics 265:977–985

Matsubayashi M (1991) Phylogenetic relationships in the potato and its related species. In: Tsuchiya T, Gupta PK (eds) Chromosome engineering in plants: genetics, breeding, evolution. Part B. Elsevier, Amsterdam, The Netherlands, pp 93–118

Malcolmson JF, Black W (1966) New R genes in *Solanum demissum* Lindl. and their complementary races of *Phytophthora infestans* (Mont.) de Bary. Euphytica 15:199–203

Michelmore RW, Meyers BC (1998) Clusters of resistance genes in plants evolve by divergent selection and a birth-and-death process. Genome Res 8:1113–1130

Meyers BC, Kozik A, Griego A, Kuang H, Michelmore RW (2003) Genome-wide analysis of NBS-LRR-encoding genes in *Arabidopsis*. Plant Cell 15:809–834

Milligan SB, Bodeau J, Yaghoobi J, Kaloshian I, Zabel P, Williamson VM (1998) The root-knot nematode resistance gene Mi from tomato is a member of the leucine zipper nucleotide binding leucine-rich repeat family of plant genes. Plant Cell 10:1307–1319

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol Plant 15:473–497

Naess SK, Bradeen JM, Wielgus SM, Haberlach GT, McGrath JM, Helgeson JP (2000) Resistance to late blight in *Solanum bulbocastanum* is mapped to chromosome 8. Theor Appl Genet 101:697–704

Park T, Vleeshouwers VGAA, Hutten RCB, van Eck HJ, van der Vossen E, Jacobsen E, Visser RGF (2005) High-resolution mapping and analysis of the resistance locus *Rpi-abpt* against *Phytophthora infestans* in potato. Mol Breed 16:33–43

Rauscher GM, Smart CD, Simko I, Bonierbale M, Mayton H, Greenland A., Fry WE (2006) Characterization and mapping of *Rpi-ber*, a novel potato late blight resistance gene from *Solanum berthaultii*. Theor Appl Genet 112:674–687

Rouppe van der Voort J, Lindeman W, Folkertsma R, Hutten RCB, Overmars H, van der Vossen E, Jacobsen E, Bakker J (1998) A QTL for broad-spectrum resistance to cyst nematode species (*Globodera spp.*) maps to a resistance gene cluster in potato. Theor Appl Genet 96:654–661

Sandbrink JM, Colon LT, Wolters PJCC, Stiekema WJ (2000) Two related genotypes of *Solanum microdontum* carry different segregating alleles for field resistance to *Phytophthora infestans*. Mol Breed 6:215–225

Sliwka J, Jakuczun H, Lebecka R, Marczewski W, Gebhardt C, Zimnoch-Guzowska E (2006) The novel, major locus *Rpi-phu1* for late blight resistance maps to potato chromosome IX and is not correlated with long vegetation period. Theor Appl Genet 113:685–695

Smilde WD, Brigneti G, Jagger L, Perkins S, Jones JDG (2005) Solanum mochiquense chromosome IX carries a novel late blight resistance gene Rpi-moc1. Theor Appl Genet 110:252–258

Song J, Bradeen JM, Naess SK, Raasch JA, Wielgus SM, Haberlach GT, Liu J, Kuang H, Austin-Phillips S, Buell CR, Helgeson JP, Jiang J (2003) Gene RB cloned from *Solanumbulbocastanum* confers broad spectrum resistance to potato late blight. Proc Natl Acad Sci 100:9128–9133

Spooner DM, van den Berg RG, Rodrigues A, Bamberg J, Hijmans RJ, Lara-Cabrera S (2004) Wild Potatoes (*Solanum* Section *Petota*) of North and Central America. Syst Bot Monogr 68:1–209

Spooner DM, van den Berg RG, Miller JT (2001) Species and series boundaries of *Solanum* series *Longipedicellata* (*Solanaceae*) and phenetically similar species in ser. *Demissa* and ser. Tuberosa: implications for a practical taxonomy of sect. *Petota*. Am J Bot 88:113–130

Stewart CNJ, Via LE (1993) A rapid CTAB DNA isolation technique useful for RAPD fingerprinting and other PCR applications. Biotechniques 14:748–750

van den Berg RG, Bryan GJ, del Rio A, Spooner DM (2002) Reduction of species in the wild potato *Solanum* section *Petota* series *Longipedicellata.* AFLP, RAPD and chloroplast SSR data. Theor Appl Genet 105:1109–1114

van der Vossen E, Sikkema A, Hekkert BL, Gros J, Stevens P, Muskens M, Wouters D, Pereira A, Stiekema W, Allefs S (2003) An ancient R gene from the wild potato species *Solanum bulbocastanum* confers broad-spectrum resistance to *Phytophthora infestans* in cultivated potato and tomato. Plant J 36:867–882

van der Vossen E, Gros J, Sikkema A, Muskens M, Wouters D, Wolters P, Pereira A, Allefs S (2005) The Rpi-blb2 gene from *Solanum* *bulbocastanum* is an *Mi-1* gene homolog conferring broad-spectrum late blight resistance in potato. Plant J 44:208–222

Vleeshouwers VGAA, van Dooijeweert W, Keizer LCP, Sijpkes L, Govers F, Colon LT (1999) A laboratory assay for *Phytophthora infestans* resistance in various *Solanum* species reflects the field situation. Eur J Plant Pathol 105:241–250

Villamon FG, Spooner DM, Orrillo M, Mihovilovich E, Perez W, Bonierbale M (2005) Late blight resistance linkages in a novel cross of the wild potato species *Solanum paucissectum* (series *Piurana*). Theor Appl Genet 111:1201–1214