Diversity, Distribution, and Evolution of *Solanum bulbocastanum* Late Blight Resistance Genes

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Submitted 18 January 2010. Accepted 5 May 2010.

Knowledge on the evolution and distribution of late blight resistance genes is important for a better understanding of the dynamics of these genes in nature. We analyzed the presence and allelic diversity of the late blight resistance genes *Rpi-blb1*, *Rpi-blb2*, and *Rpi-blb3*, originating from *Solanum bulbocastanum*, in a set of tuber-bearing *Solanum* species comprising 196 different taxa. The three genes were only present in some Mexican diploid as well as polyploid species closely related to *S. bulbocastanum*. Sequence analysis of the fragments obtained from the *Rpi-blb1* and *Rpi-blb3* genes suggests an evolution through recombinations and point mutations. For *Rpi-blb2*, only sequences identical to the cloned gene were found in *S. bulbocastanum* accessions, suggesting that it has emerged recently. The three resistance genes occurred in different combinations and frequencies in *S. bulbocastanum* accessions and their spread is confined to Central America. A selected set of genotypes was tested for their response to the avirulence effectors IPIO-2, Avr-blb2, and Pi-Avr2, which interact with *Rpi-blb1*, *Rpi-blb2*, and *Rpi-blb3*, respectively, as well as by disease assays with a diverse set of isolates. Using this approach, some accessions could be identified that contain novel, as yet unknown, late blight resistance factors in addition to the *Rpi-blb1*, *Rpi-blb2*, and *Rpi-blb3* genes.

Solanum bulbocastanum is well known for its resistance to late blight (Niederhauser and Mills 1953), a disease caused by the oomycete *Phytophthora infestans*. The species belongs to the section *Petota* of the genus *Solanum*. It is a diploid, self-incompatible species native to Central America. It inhabits mountainous areas at an altitude between 1,200 and 2,300 m (Spoonier et al. 2004) from northwestern Mexico (Durango and Nayarit regions) all the way south to Honduras.

Although characterized by late blight resistance, *S. bulbocastanum* resistance sources have not been widely exploited

yet, which is mainly due to crossing barriers with cultivated potato (Hermsen and Ramanna 1973; Helgeson et al. 1998). Until now, four different nucleotide-binding site leucine-rich repeat resistance genes have been identified in and cloned from *S. bulbocastanum*: namely, *Rpi-blb1* (van der Vossen et al. 2003), also known as *RB* (Song et al. 2003); *Rpi-blb2* (van der Vossen et al. 2005); *Rpi-blb3* (Lokossou et al. 2009); and *Rpi-bt1* (Oosumi et al. 2009), which opens the possibility to study the distribution and diversity of these genes in the section *Petota*.

The distribution of specific resistance genes in natural populations has been studied in a few cases only: *Rps2* and *Rpp13* in *Arabidopsis thaliana* (Mauricio et al. 2003; Rose et al. 2004), *Cf-4* and *Cf-9* in tomato (Kruijt et al. 2005; Caicedo 2008), as well as *Pm3* (Bhullar et al. 2009; Yahiaoui et al. 2009) and *Lr21* (Huang et al. 2009) from wheat. Mauricio and associates (2003) studied the diversity of *Rps2*, a resistance gene against the bacteria *Pseudomonas syringae*, in a world-wide sample of 27 *A. thaliana* accessions. They favored balancing selection as the explanation for the *Rps2* variation. This maintenance of variance by natural selection might be a general feature for the dynamics of resistance (*R*) gene evolution (Ellis et al. 1999; Luck et al. 2000). From the study on diversity for *Rpp13*, a resistance gene in *A. thaliana* against *Hyaloperonospora arabidopsis*, which is characterized by an extreme sequence diversity and resistance to various pathogen isolates, the suggestion was made that the co-evolutionary interaction between host and pathogen must be very active (Rose et al. 2004). Confirmation was provided with the analysis of the recently identified avirulence (*Avr*) gene of *Rpp13*, *Atr13*, which reveals high sequence diversity for this effector as well (Allen et al. 2008); moreover, one *Rpp13* allele seems to recognize another *H. arabidopsis* effector (Hall et al. 2009). Kruijt and associates (2005) showed that a high proportion of wild tomato plants specifically recognize the *Cladosporium fulvum* Avr4 and Avr9 elicitors, and functional homologs of the *C. fulvum* resistance genes *Cf-4* and *Cf-9* are conserved in diverged tomato species. The *Cf-4* and *Cf-9* genes appear to have been maintained by selection pressure imposed by the pathogen *C. fulvum*. Caicedo (2008) studied the distribution of *Cf-2* homologs in natural populations of *S. pimpinellifolium* and deduced that, primarily, the geographic distribution of *Cf-2* homolog diversity has been shaped by demographic factors or by selective pressure with a clinal geographic distribution. Levels of homologue diversity varied greatly between populations. Huang and associates (2009) investigated the origin of

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*The e-Xtra logo stands for “electronic extra” and indicates that four supplementary tables and one supplementary figure are published online.

the leaf rust resistance gene *Lr21* that has been introgressed into hexaploid wheat from *Aegilops tauschii* by functional screens and sequence analysis. The *Lr21* gene was found in only 12 accessions out of the 528 accessions representing the geographic diversity of the goatgrass *Aegilops tauschii*. All 12 had identical sequences and had been collected within a small area along the Caspian sea in Iran and Azerbaijan. Bhullar and associates (2009) investigated the distribution of the *Pm3* gene, which confers resistance to the powdery mildew fungus in wheat, in a selected set of 1,320 bread wheat landraces. Additionally, Yahiaoui and associates (2009) showed that the *Pm3* sequence diversity was higher in wild emmer wheat from the Levant area compared with the accessions from Turkey.

As population genetic processes and dynamics might affect the evolution of *R* genes, a better understanding of variation within and between populations is crucial. Spooner and associates (2009) evaluated several factors that might be predictive for identifying pest- and disease-resistant plants in wild relatives of the cultivated potato. For late blight resistance, they identified taxonomic, climatic, and geographic (latitude and altitude) variables. From these analyses, it was clear that species level was the best predictor for resistance against *Phytophthora infestans*. These results stressed the need to use more focused evaluations of germplasm and to have a clear picture of geographical patterns of diversity in late blight resistance genes in wild *Solanum* species. To analyze these, here, use has been made of well-studied *R* genes such as *Rpi-blb1*, *Rpi-blb2*, and *Rpi-blb3*, which are excellent candidates because both *Avr* and *R* genes have been identified and cloned.

The *Rpi-blb1* gene is located on linkage group VIII near marker CT64 in a cluster of four resistance gene analogues (RGA) (van der Vossen et al. 2003). Its susceptible haplotype, *rb*, is characterized by an 18-bp deletion (Song et al. 2003). In an evaluation by Wang and associates (2008) using 44 species of *Solanum* section *Petota* for *Rpi-blb1* (RGA2) and its flanking RGA (RGA1 and RGA3), it was shown that the genes had a different distribution and diversity. The flanking RGA1 appeared to be highly conserved within the tuber-bearing *Petota* and was also found in the non-tuber-bearing species of the section, while RGA3 was only present in some tuber-bearing species. The *Rpi-blb1* gene was found in Mexican diploid species (*S. bulbocastanum* and *S. cardiophyllum*) and the Mexican polyploid species *S. stoloniferum* (including *S. papita* and *S. polytrichon*) (Wang et al. 2008). Functional analysis of the genes found in *S. stoloniferum* (*Rpi-sto1* and *Rpi-pta1*) indeed confirmed the functional homology with *Rpi-blb1* (Vleeshouwers et al. 2008). The occurrence of *Rpi-blb1* homologues in *S. stoloniferum* was suggested to be the result of common ancestry (e.g., *S. bulbocastanum* was suggested as one of the parents of the allopolyploid *S. stoloniferum*) (Wang et al. 2008).

Rpi-blb2 is located on linkage group VI near marker CT119, in a locus harboring at least 15 *Mi-1* gene homologues (van der Vossen et al. 2005). The *Mi-1* gene of tomato confers resistance to the root-knot nematode *Meloidogyne incognita* (Milligan et al. 1998) and shares 82% overall protein sequence identity with *Rpi-blb2* (van der Vossen et al. 2005). *Rpi-ver1*, a late blight *R* gene from *S. verrucosum*, also maps in this region (Jacobs et al. 2010). Other *R* genes found at this locus in tomato include *Ol-1* and *Ol-3* (Bai et al. 2005) and *Cf2* and *Cf5* (Dickinson et al. 1993), conferring resistance to *Oidium neoly copersici* and *C. fulvum*, respectively. Apart from *S. bulbocastanum*, no other species were found to contain *Rpi-blb2* (Wang et al. 2008).

Rpi-blb3 is located on linkage group IV near marker TG339 in a major late blight resistance gene cluster containing *R2*, *Rpi-abpt*, and *R2-like* (Park et al. 2005). Late blight resistances with a more quantitative trait locus (QTL)-like appearance such as those of *S. microdontum* (Sandbrink et al. 2000; Tan et

al. 2008) and those found in tomato (Brouwer et al. 2004) map into the same region. Tan and associates (2008) proposed that the QTL from *S. microdontum*, identified as *Rpi-mcd1*, is a major *R* gene. Other *R* genes, mapping to the same region, include *Ny_{tblr}*, conferring resistance to *Potato virus Y* (Celebi-Toprak et al. 2002), and the *Hero* resistance gene which confers resistance to all known pathotypes of *Globodera rostochiensis* and partial resistance to *G. pallida* (Ernst et al. 2002). Allele mining studies for *Rpi-blb3* are not yet reported.

Distribution of *R* genes in natural populations is not easy to study phenotypically, as resistance to a particular pathogen might result from the presence of one or more different *R* genes, which is the case for populations of *S. bulbocastanum*. One approach could be to use *Avr* genes to determine whether a corresponding *R* gene is present; in our case, *Avrblb1*, which interacts with *Rpi-blb1* (Vleeshouwers et al. 2008); *Avrblb2*, interacting with *Rpi-blb2* (Oh et al. 2009); and *Pi-Avr2*, interacting with *Rpi-blb3* (Lokossou et al. 2009). However, the *Rpi-blb3* gene has several close relatives, all recognizing the same set of *Avr* genes (Lokossou et al. 2009). Also, carrying out such analysis in a large number of different species is not straight forward. Studying the distribution of *R* genes with highly specific molecular markers and deduction of the phenotype for positive genotypes is an alternative. However, one has to keep in mind that the functionality of *R* genes might be changed by one single-nucleotide polymorphism (SNP) or by insertions or deletions. A combination of the two approaches and screening with key *P. infestans* isolates seems to be a beneficial strategy that will allow the identification of other resistance genes toward this devastating disease.

In this article, we studied the allelic frequencies of the *Rpi-blb1*, *Rpi-blb2*, and *Rpi-blb3* genes in several wild tuber-bearing *Solanum* accessions by performing allele mining with *R*-gene-specific primers. Additionally, *Avr* expression assays as well as detached leaf assays with key *P. infestans* isolates were used to strengthen the evidence for the presence of functional alleles. We relate the occurrence of the genes to the geographical origin of the accessions.

RESULTS

Screening of *Solanum* section *Petota* for the presence of *Rpi-blb* resistance genes.

In total, 196 different taxa from *Solanum* section *Petota* were screened for the presence of the *Rpi-blb1* and *Rpi-blb3* genes. The presence of *Rpi-blb2* was not assessed in this wide set of taxa because a previous study had already shown that the distribution of this gene was confined to *S. bulbocastanum* (Wang et al. 2008). We used the plant material described by Jacobs and associates (2008) and, as far as available, at least five accessions from each species and five individuals per accession (in total, 4,929 genotypes). One screen was carried out with the 1/1' primer pair, which is specific for the functional allele of *Rpi-blb1* (Colton et al. 2006). Polymerase chain reaction (PCR) fragments were detected in *S. bulbocastanum*, *S. cardiophyllum* subsp. *cardiophyllum*, and *S. stoloniferum* (sensu Spooner et al. 2004) only.

The collection was also screened with primers specific for *Rpi-blb3*, and six species were found to contain putative *Rpi-blb3* homologues: *S. pinnatisectum*, *S. bulbocastanum* (including some subspecies), *S. hjertingii*, *S. nayaritense*, *S. brachistotrichum*, and *S. stoloniferum*.

Detailed investigation of *S. bulbocastanum* and related species.

An additional 72 accessions originating from Mexico and Guatemala of the series *Bulbocastana*, *Pinnatisecta*, and *Longi-*

pedicellata was evaluated for the presence of *Rpi-blb1*, *Rpi-blb2*, and *Rpi-blb3* genes. For 63 accessions, precise coordinates (longitude and latitude data) were available, pointing to 52 different locations (Supplementary Table S1).

blb3 fragments were found together in two *S. bulbocastanum* accessions from two distinct locations (Fig. 1B).

Allelic variation
in the *Rpi-blb1*, *Rpi-blb2*, and *Rpi-blb3* gene fragments.

types without nonsynonymous SNP and is only composed of *S. bulbocastanum* accessions with a sequence identical to the cloned *Rpi-blb1* gene. Group B additionally contains *S. stoloniferum* (*Rpi-stol* like) haplotypes while *S. papita* and *S. polytrichon* (*Rpi-ptal* and *Rpi-plt1* like) are part of group C. Haplotypes C2, A11, C6, E2, I1, and L1 display double SNP which are probably the result of heterozygous genotypes. Haplotype M1 represents the known nonfunctional allele, identified by the 18-bp deletion (Song et al. 2003).

clearly defining a polymorphic sequence block. The B haplotypes are exclusively identified in accessions originating from Guatemala (Supplementary Fig. 1). Haplotype B6 contains a SNP in position 1,396, resulting in a stop codon. Group C comprises haplotypes with four SNP in common also defining a polymorphic sequence block, present in *S. brachistotrichum*, *S. nayaritense* (Table 4; Supplementary Table S4), and *S. stoloniferum*. The R2-like, *Rpi-abpt*, and R2 alleles are classified in haplotypes D1 and E1.

In an attempt to link the presence of *Rpi-blb* haplotypes to late blight resistance, a set of 50 plants from our in vitro collection was screened. It included a molecular characterization by PCR amplification with *Rpi-blb1*, *Rpi-blb2*, and *Rpi-blb3* gene-specific primers followed by sequencing the PCR fragment. An additional combination of *Rpi-blb3* primers, Blb3 F/Ra, was used, with Ra being specific to *Rpi-blb3* and its homolog *R2-like* (Lokossou et al. 2009), in order to pick up intermediates of *Rpi-blb3* and *R2-like*. Phenotypic characterization was carried out through detached leaf assays with six *P. infestans* isolates (Supplementary Table S2) and agroinfiltration with Pi effectors interacting with *Rpi-blb1* and *Rpi-blb3*. The chosen *P. infestans* isolates were genetically very different from each other, as has been shown by simple-sequence repeat

Table 1. Allelic variation for *Rpi-blb1* in *Solanum bulbocastanum* accessions and related species^a

^a The *Rpi-blb1* sequence spans 1,709 bp in total, composed of two separate parts: i) from the start codon to position 520, derived from the primer 517/1519, with polymorphic nucleotides located in the intron sequence in italics; and ii) from position 2,404 to the stop codon, derived from the primer BLB1F/R and 1521/519.

Haplotype	SNP position in comparison with <i>Rpi-blb2</i> genomic sequence																							Accessions
	2226-2297																							
A	CTGTGAAGAAAGTGGTAAAAATTATTGGATGATTAAATTCAGTAGCTGGTAATTGTTCAATGAGAT																							<i>Rpi-blb2</i> , BLB-CGN17692, BLB-960631, BLB-P1275195, BLB-P1498011, BLB-P1498223, BLB-CPH-CGN18326, CPH-CGN22387 CPH-CGN18326, CPH-CGN22387
B	-																							

^a The *Rpi-blb2* sequence spans 715 bp derived from the primers Blb2F/R.

fingerprinting (Champouret et al. 2009), and displayed different virulence spectra with respect to the *Rpi-blb* genes tested. The *Rpi-blb2* gene conferred resistance to the six isolates used and the *Rpi-blb1* and *Rpi-blb3* gene conferred the resistance

spectrum shown in Table 4. None of the tested plants contained *Rpi-blb2*, because no fragment was amplified with the primers Blb2F/R. Unfortunately, we observed background responses caused by the agroinfiltration of Avrblb2 in all the

Table 3. Allelic variation for *Rpi-blb3* in *Solanum bulbocastanum* accessions and related species^a

Haplotype	SNP position in comparison with <i>Rpi-blb3</i> genomic sequence																																Accessions			
	941	972	1001	1016	1021	1027	1026	1036	1043	1045	1065	1103	1107	1116	1131	1133	1196	1235	1239	1255	1258	1272	1293	1309	1326	1338	1352	1371	1396	1439	1487	1501		1510		
	G	T	G	T	G	G	G	A	G	T	A	G	A	G	A	A	T	C	G	C	T	T	G	C	C	G	C	G	C	C	C	C	T	G		
A 1																																			PNT-CGN17745, PNT-CGN17740, <i>Rpi-blb3</i> , DPH-CGN17688, PTT-CGN23074, BLB-PI545711, BLB-CGN17690, BLB-CGN17687, HJT-CGN22370, HJT-CGN18345, PNT-CGN17742, STO-CGN18348, STO-CGN18333	
A 2																				a															BLB-CGN22698, BLB-CGN23010, BLB-PI 275185, BLB-BGRC53682, BLB-PI275190, BLB-PI310960, BLB-PI347757, BLB-PI498224, BLB-PI498225, BLB-PI545711, BLB-PI545751, BLB-CGN176888, DPH-PI253210, BST-GLKS32717, PTT-CGN23074, BLB-GLKS31741	
A 3																																			BLB-PI590930	
A 4																																			DPH-CGN17688	
A 5																																			PTT-CGN17689	
A 6																																			DPH-CGN17688	
A 7																																			BLB-BGRC53682	
A 8																																			PTT-PI275200	
B 1																									a		t								PTT-CGN23074	
B 2												a			a									a		t									PTT-CGN21364, BLB-PI604065, PTT-CGN21363	
B 3												a			a									a		t									BLB-CGN18310	
B 4															a									a		t									PTT-CGN22732	
B 5															a									a		t									PTT-CGN22732	
B 6												C			a									a		t									BLB-CGN18311, PTT-CGN23075	
B 7												C			a									a		t									PTT-CGN23074	
B 8															a									a		t									PTT-CGN21363	
C 1																																			STO-PI205522	
C 2																																			BST-CPC3822, BST-PI320265	
C 3																																			BST-PI320265	
C 4																																			NYR-PI545825	
D 1																																				<i>R2-like, Rpi-abpt</i>
E 1																																			<i>R2</i>	
Amino acid change	R/I	S/S	R/K	V/A	A/S	V/I	M/I	M/L	S/N	L/L	E/D	K/K	G/G	S/N	fs	fs	I/N	T/S	A/A	I/L	Y/H	F/L	K/K	R/W	E/E	P/P	R/I	D/E	R/stop	T/S	A/V	F/L	V/I			

^a The *Rpi-blb3* sequence spans 618 bp derived from the primers Blb3F/R. The nucleotides of *Rpi-blb* gene sequences are in capital letters. Haplotype codes are indicated in the left column. Nucleotide positions are numbered according to the genomic sequences of the genes, beginning at the START codon and including introns in the case of *Rpi-blb1* and *Rpi-blb2*. Amino acid changes are indicated on the bottom line. Single-nucleotide polymorphism (SNP) portions of the intron region are in italics. The position where SNPs did not change the amino acid are highlighted in light gray. SNPs resulting in nonsynonymous amino acid changes are indicated in bold. *Solanum* accessions in which the haplotypes were identified are indicated on the right column. Nucleotide abbreviations according to the IUB code are in capital letters: K, GT; M, AC; R, AG; S, GC; W, AT; Y, CT.

Table 4. Evaluation of representative *Solanum* genotypes by molecular characterization, effector agroinfection, and detached-leaf assay^a

Taxon	Accession number	Specific amplification			Sequence		Effector Agro-infiltration				Phytophthora infestans Detached leaf assay							Comments
		Rpi-blb1 1/1'	RpiB3 F/R	RpiBlb3 F/Ra	RpiBlb1 Haplotype	RpiBlb3	Avr-blb1	Pi-Avr2	Controls		PIC99177	PIC99189	PIC99183	IPO-C	90128	UK7824		
									R3a	R3a/Avr3a								
BLB	CGN17692 (Blb8005-8)	x		x	A1	+++	++	-	+++		S	S	R	R	R	R	Genotype containing Rpi-blb1	
DPH	CGN17688 (Blb99-256-3)		x	x	A1	-	+++	-	+++		Q	S	R	S	R	S	Genotype containing Rpi-blb3	
HJT	CGN22370		x		A1	-	+++	-	+++		S	S	R	S	R	S		
HJT	BGRC32671		x		A1	-	+++	-	+++		S	S	R	Rq	S	R	S	
HJT	CGN17717			x	A1	-	+++	-	+++		R	S	R	S	R	S		
STO	CGN18333		x		A1	-	+++	-	+++		S	R	R	S	R	S		
PNT	CGN18331		x	x	A1	+	+	-	+++		R	R	R	R	R	R	Plants with Avr2 recognition and Rpi-blb3 fragment	
BST	CGN17681		x	x	A2	-	+++	-	+++		S	S	R	S	Rq	S		
BLB	CGN18310		x		B2	-	++	-	+++		R	R	R	Q	R	R		
PTT	CGN21364				B2	-	+	-	+++		R	Q	R	R	R	R		
BST	PI320265			x	C2	-	+++	-	++		S	R	R	S	R	Q		
NYR	PI545825		x		C4	-	+++	-	+++		Q	R	R	R	S	R	R	
STO	PI205522	x	x		A9	C1	+++	+++	-	+++	Q		R	R	R	R	Plant with Avr2 and AvrBlb1 recognition and Rpi-blb1 and Rpi-blb3 fragments.	
BLB	CGN17689	x	x		A1	A5	+++	-	-	+++	R	R	R	R	R	R	Plants with AvrBlb1 recognition and Rpi-blb1	
STO	CGN18346			x	A1		+++	-	-	+	R	S	R	R	R	R	Plants with AvrBlb1 recognition and no Rpi-blb1 fragment.	
BLB	CGN17693						+	-	-	+	R	R	R	R	R	R		
STO	CGN18333		x	x	A1		-	-	-	++	Q	R	S	S	R	R	Plants with Rpi-blb1 and/or Rpi-blb3 fragments	
BLB	BGRC53682	x	x	x	A5	A2	-	-	-	+++	R	S	R	R	R	R	but without Avrblb1 or Pi-Avr2 recognition.	
BLB	CGN23010						-	-	-	+++	R	R	R	R	R	R	Plants containing different late blight resistance genes other than the tested Rpi-blb genes.	
CPH	BGRC55227						-	-	-	+++	Q	Q	S	R	S	R		
PTT	CGN23074		x		B1	nd	-	-	-	++	S	S	S	S	S	S	Late blight susceptible plants	
BST	CPC3822		x	x			-	+++	-	+++	S	S	S	S	S	S		
PNT	CGN17743			x			+	++	++	++	R	R	R	R	R	R	Plants not suitable for agro-infiltration	
BLB	CGN22367			x			-	-	-	-	R	Q	R	R	R	R		

^a *Solanum* accession taxa are indicated in the column from left to right; identification number (genotype number in bracket); results of the specific amplification of *Rpi-blb1* by the primers combination 1/1' and *Rpi-blb3* by Blb3 F/R and Blb3 F/Ra with Ra being specific to *Rpi-blb3* and *R2-like* (Lokossou et al. 2009); the haplotype identified after sequencing of the specific fragment (referring to Table 1); results of the effector agroinfection with Avr-blb1, Pi-Avr2, and the controls R3a and Pi-Avr3a/R3a together, are indicated by degree of response intensity from – = no response to +++ = clear hypersensitive reaction; results of the detached leaf assay conducted with the isolates PIC99177, PIC 99189, PIC 99183, IPO-C, 90128, and UK7824 scored as R (resistant), S (susceptible), Rq (quantitatively resistant), and Q (quantitative). Abbreviations: ND, sequences not determined; BLB, *S. bulbocastanum*; BST, *S. brachistotrichum*; CPH, *S. car-diophyllum*; DPH, *S. bulbocastanum* subsp. *dolichophyllum*; FEN, *S. fendleri*; HJT, *S. hjertingii*; NYR, *S. nayaritense*; PLT, *S. polytrichon*; PNT, *S. pinnatisec-tum*; PTA, *S. papita*; PTT, *S. bulbocastanum* subsp. *partitum*; STO, *S. stoloniferum*. CPH-BGRC55227 contains *Rpi-blb2* haplotype B.

plants tested (not shown); thus, *Rpi-blb2* presence or absence could not be verified by effector recognition in wild *Solanum* spp. Results obtained for *Rpi-blb1* and *Rpi-blb3* are presented in Table 4 and show the diversity of possible interactions, classified in five categories.

The first category comprises plants harboring one or both of the *Rpi-blb1* and *Rpi-blb3* genes, as judged from the marker analysis. They interacted with one or both of the Avrblb1 and Pi-Avr2 elicitors and displayed a resistance spectrum identical to or broader than the *S. bulbocastanum*-derived *R* genes. Based on the assay with Avrblb1, three *S. bulbocastanum* and one *S. stoloniferum* plants contained *Rpi-blb1*. PCR amplification and sequencing strengthened the presence of functional *Rpi-blb1* haplotypes, identified as A1 and A9. Their resistance spectrum matched with that of an *S. bulbocastanum* plant (CGN17692), which was confirmed to harbor *Rpi-blb1* (van der Vossen et al. 2003). However, they were resistant to isolates PIC99189 and PIC99177, known to break *Rpi-blb1* (Champouret et al. 2009), which suggests the presence of additional resistance factors in these genotypes. The *Rpi-blb3* haplotypes A1, B2, C1, C2, C3, and C4 were found in 14 plants from *S. bulbocastanum*, *S. brachistotrichum*, *S. hjertingii*, *S. nayaritense*, and *S. stoloniferum* accessions. They all interact with Pi-Avr2 and display an identical or broader resistance spectrum compared with the progenitor used to isolate *Rpi-blb3*, thus suggesting the presence of a functional *Rpi-blb3* haplotype and additional late blight resistance factors.

From plants classified in the second category, fragments were amplified with primers specific for one or both *Rpi-blb1* and *Rpi-blb3* genes but no interaction with Avrblb1 or Pi-Avr2 was observed. The *Rpi-blb* genes amplified might contain mutations preventing the interaction with the tested effectors. Moreover, these plants putatively contain late blight resistance genes other than the one tested, as judged from the responses to the isolates (Table 4).

The third category comprises resistant plants lacking *Rpi-blb1* or *Rpi-blb3*, as judged from the marker assays and effector interaction. Interestingly, two *S. bulbocastanum* and two *S. cardiophyllum* plants featured complete resistance to the full set of isolates. These plants probably contain late blight resistance genes other than the tested *Rpi-blb* genes. One *S. cardiophyllum* plant (CPH-BGRC55227) tested was positive for *Rpi-blb2* haplotype B and displays a resistance spectrum different from that of a *Rpi-blb2*-containing plant.

Late-blight-susceptible plants not containing any functional *R* gene, as judged from the response with the tested isolates, but showing amplification with *Rpi-blb3* primers are found in category 4. One *S. brachistotrichum* clone contained *Rpi-blb3* haplotype C2 and interacted strongly with Pi-Avr2 but was completely susceptible to the set of *P. infestans* isolates tested. Three accessions from *S. bulbocastanum* subsp. *partitum*, two of *S. brachistotrichum*, and one of *S. stoloniferum* contained *Rpi-blb3* haplotypes B1, B2, B7, and C2 but did not interact with Pi-Avr2 and displayed a susceptible phenotype when infected with the set of isolates.

The last category of plants was not suitable for agroinfiltration. The reactions with the effectors were nonspecific and resulted in a hypersensitive response to all the tested effectors or gave no reaction to control Pi-Avr/R gene co-infiltration (Table 4). However, plants showing resistance to all tested isolates are likely to contain new late blight resistance gene.

Geographical distribution of the *Rpi-blb* genes.

The three *Rpi-blb* genes were mainly present in the *S. bulbocastanum* accessions from Central Mexico (Fig. 2) and plants not containing the three studied *Rpi-blb* genes originated from the northern part of Mexico and the southern part of the United

States. Also, the allotetraploid species *S. fendleri*, which is found in the north of Mexico, lacks the *Rpi-blb* genes tested.

S. stoloniferum from the Durango region contained *Rpi-blb1* whereas *Rpi-blb3* was present in *S. hjertingii* originating from Monterrey, *S. brachistotrichum* from Chihuahua, and *S. bulbocastanum* subsp. *partitum* from Guatemala.

In order to see whether haplotypes diverge when they originate from more geographically distant areas, the haplotypes were phylogenetically mapped in Google Earth using Supramap (Janies et al. 2007). From the results, it can be seen that the M1 haplotype of *Rpi-blb1* is present in Central Mexico as well as in Guatemala and that the less-supported clade containing the C and B haplotypes from *S. stoloniferum* is confined to the upper part of Mexico. For *Rpi-blb3*, the haplotypes of group C originate from Central toward Northern Mexico, whereas haplotypes recognized as group B all are from Guatemala.

DISCUSSION

The use of gene-specific primers and gene functionality.

Gene-specific primers are powerful tools for the evaluation of the presence or absence of resistance genes (Arens et al. 2010). Subsequent sequencing of the fragment makes it possible to verify their identity, differentiate the haplotypes, and discard recognized pseudogenes, thus indicating the presence or absence of the most likely functional genes. We validated this approach by phenotyping 50 individual plants using effectors and detached leaf assays. The plants were also characterized for the presence of *Rpi-blb1*, *Rpi-blb2*, and *Rpi-blb3* with markers. In the 50 cases, we only observed two cases for *Rpi-blb1* in which the markers predicted the presence of the gene where there was no effector response. This indicates that there is a good but not 100% correlation between markers and phenotype for *Rpi-blb1* (Table 4). All plants tested negative when using the gene-specific markers for *Rpi-blb2*. Unfortunately, this could not be validated by an effector response due to background that was observed in the agroinfiltration experiment with Avrblb2. For *Rpi-blb3*, we found 13 cases in which there was amplification with the gene-specific primers but no response to the effector. There were three cases in which there was a response to the effector but no amplification of the gene-specific fragment and one case in which we observed an effector response and no resistance in the detached leaf assay. An additional primer pair (Blb3 F/Ra) did amplify in some of the cases where there was no amplification with the *Rpi-blb3*-specific primers was found (Table 4), suggesting that similar but slightly different genes are present. This was clear from the sequence analysis of some of the fragments obtained with the *Rpi-blb3*-specific primers (e.g., the B and C haplotypes of *Rpi-blb3*). These results reflect the complexity of the *Rpi-blb3* gene family and underline the usefulness of the combined techniques to identify *Rpi-blb3* from the other alleles of the cluster. However, it should be stressed that, in most cases where there was a discrepancy observed between marker assays and phenotypic assays, this was outside *S. bulbocastanum*.

Discrepancies between molecular and functional assays can have several causes. For example, plants containing an *R* allele-specific fragment are for some reason unable to interact with the Avr-protein. In such cases, the *R*-gene fragment found is probably not translated into a protein interacting with the Avr protein, as was also observed by Hall and associates (2009) in *H. arabidopsidis*. Also, recognition specificity of different effectors by different alleles may be an important factor, as was previously demonstrated for the *RPM1* locus of *Arabidopsis thaliana* (Bisgrove et al. 1994; Grant et al. 1995), the *L* locus in flax (Dodds et al. 2004), and the *Pto* locus in tomato (Kim et al. 2002; Ronald et al. 1992).

Distribution of *Rpi-blb* genes in *Solanum* spp.

Our study provides a clear picture of the distribution of late blight resistance genes so far isolated from *S. bulbocastanum*. It shows that *Rpi-blb1*, *Rpi-blb2*, and *Rpi-blb3* have a very limited distribution among species. Their spread is restricted to the Mexican diploid species and the polyploid Longipedicellata group and confined to Central America. The occurrence of similar haplotypes in different diploid species may be explained by the fact that these species are able to form interspecific hybrids, especially *S. bulbocastanum* and *S. cardiophyllum* (Graham 1965). The presence of both *Rpi-blb1* and *Rpi-blb3* in the polyploid Longipedicellata may be explained by the fact that this group of *Solanum* section *Petota* is of allotetraploid origin. It has been shown that *S. hjertingii*, for example, consists of an *S. verucosum*-like and an *S. cardiophyllum*-like genome (Pendinen et al. 2008). Wang and associates (2008) suggested that *S. bulbocastanum* may be one of the parental species of the allotetraploid *S. stoloniferum*. The observation that the late blight resistance genes evaluated are confined to Central America might be true for the distribution of other *R* genes as well.

Rpi-blb genes are found in different frequencies in *S. bulbocastanum* populations.

Gene bank accessions used in this study consist of plant material that has been generatively multiplied by the gene bank

prior to usage. In the Netherlands, at the Centre for Genetic Resources (CGN), this is done without any *P. infestans* pressure, because protective spraying is applied every week. This might have resulted in slight frequency changes in the presence of the *Rpi-blb* genes compared with the actual situation in Mexico.

We assessed the frequency in which the different genes are present in the *S. bulbocastanum* accessions under study. Individual plants containing all three *Rpi-blb* genes have been found at very low frequency (less than 1%) and in two *S. bulbocastanum* accessions only, probably because of the rarity of *Rpi-blb2*. The *Rpi-blb1* and *Rpi-blb3* genes have been detected together in 27% of the *S. bulbocastanum* plants analyzed (52% of the accessions). Only in *S. bulbocastanum* PI498225 did all individual plants contain *Rpi-blb1* and *Rpi-blb3*, suggesting that accumulation of these genes in *S. bulbocastanum* is a rare event. On the other hand, our functional test with the 50 plants frequently showed a broader resistance spectrum than expected with the tested *Rpi-blb* genes, indicating the presence of other, unknown, *Rpi* genes.

Almost all the accessions tested contain putative resistant and susceptible individuals. This might reflect a strongly fluctuating *P. infestans* pressure.

The susceptible haplotype of *Rpi-blb1*, denoted as M1, is abundantly present and scattered around in Central America.

Fig. 2. Geographical location of the *Solanum* accessions under study containing only *Rpi-blb1* (triangles), only *Rpi-blb2* (squares), only *Rpi-blb3* (white-filled dots), *Rpi-blb1* and *Rpi-blb3* (lozenges with a white center), *Rpi-blb1* and *Rpi-blb2* (stars), the three *Rpi-blb* genes (cross), and the accessions not containing any *Rpi-blb* gene (solid black dots).

Long-lived coexistence of resistant and susceptible alleles has been observed in other species as well, such as *A. thaliana* (Henk et al. 1999; Stahl et al. 1999) and wheat (Yahiaoui et al. 2006), and may be related to plant fitness in the absence of selective pressure (Tian et al. 2003; McDowell and Simon 2006; Kramer et al. 2009).

Evolution of *Rpi-blb* genes.

Sequence analysis of the fragments obtained for the *Rpi-blb1* and *Rpi-blb3* genes suggests an evolution through recombination and point mutations. In this respect, there are clear differences between the two genes. *Rpi-blb1* is thought to have resulted from a recombination event involving *RGA1-blb* and *RGA3-blb* (van der Vossen et al. 2003), and sequence analysis (Table 1) suggests a further evolution through point mutations, an evolutionary pattern typical for a type II resistance gene as described for *R* genes in natural lettuce populations (Kuang et al. 2004).

The situation with *Rpi-blb3* is more complex than with *Rpi-blb1* and most likely related to the complex nature of the cluster in which the gene is present. In this cluster, several genes have almost identical sequences (Lokossou et al. 2009). Although all sequence information available has been taken into account for the design of the gene-specific primers for *Rpi-blb3*, it cannot be excluded that some variants may not be discriminated by these primers. In *Solanum* spp. as a whole, even more *Rpi* genes have been mapped to the *Rpi-blb3* cluster. Examples are *Rpi-dem1* from *S. demissum* (Hein et al. 2007), *Rpi-snk1* (Jacobs et al. 2010), and QTLs from *S. microdontum* (Colon et al. 1995; Micheletto et al. 2000; Sandbrink et al. 2000). Such resistances might represent *R* genes which are homologues or recombinants sharing ancestry with *Rpi-blb3* as well as *R* genes that have evolved independently and interact with other or the same effectors. In the same way, functional *Pm3* resistance genes independently evolved in wild tetraploid wheat and domesticated bread wheat (Yahiaoui et al. 2009).

The presence of conserved SNP patterns among the haplotypes suggests independent recombinations between ancestors of *Rpi-blb3*. In wheat, recombination has been shown to have resulted in the birth of the *Lr21* resistance gene (Huang et al. 2009). This gene was found to be a chimera between two non-functional haplotypes.

Recent studies show that the recombination level at *R* clusters is increased following pathogen infection, suggesting a mechanism that induces temporary genome instability in response to extreme stress (Lucht et al. 2002; Kovalchuk et al. 2003). This instability might well be connected to the high intensity of transcription of some *R* genes seen just after infection (Kramer et al. 2009), in which the genome must be temporarily accessible for efficient transcription. It is conceivable that, depending on the disease pressure, we have a fast pattern of evolution of *R*-gene clusters dominated by sequence exchange or a slow pattern of evolution dominated by point mutations. Both evolutionary mechanisms appear to have shaped the *Rpi-blb1* and *Rpi-blb3* clusters. Additionally, the distinction between type I and II seems rather artificial; empirical sequence data suggests a continuum between the two types. Likewise, comparisons of sequences obtained through allele mining conducted at the *Pm3* locus in wheat (Bhullar et al. 2009) show differences that originated from point mutations and illegitimate recombination.

The absence of mutation or recombination in the *Rpi-blb2* fragment amplified from *S. bulbocastanum* and the low percentage of *Rpi-blb2*-containing plants show that this gene emerged recently and didn't evolve through point mutations yet.

New *P. infestans* *R* genes identified.

In our analysis, 76% of late-blight-resistant genotypes cannot be explained by only the presence of the *Rpi-blb1*, *Rpi-blb2*, and *Rpi-blb3* genes. Clear examples are *S. bulbocastanum* accessions CGN23010 and P1275199; however, genotypes from CGN17689 and CGN21306 also contain additional resistance genes, because their resistance spectrum is different from *Rpi-blb1*, *Rpi-blb2*, or *Rpi-blb3*. This confirms that *S. bulbocastanum* is a rich source of late blight resistance genes, as indicated by Budin (2002). The *S. stoloniferum* accession PI205522, containing *Rpi-blb1* and *Rpi-blb3*, based on the marker assays, contains an additional gene based on the resistance spectrum observed (Table 4). The gene involved is probably a paralog of R4^{MA} (Van Poppel et al. 2009) because it confers resistance to isolate PIC99189 (avirulent on R4^{MA}) and a hypersensitive response with Avr4 effector (van Poppel et al. 2008) was found (*unpublished results*). The possibility cannot be excluded that the recently described gene *Rpi-bt1* (Oosumi et al. 2009) is responsible for some of the resistant phenotypes found in *S. bulbocastanum*. Because we found *Rpi-blb1* and *Rpi-blb3* in species related to *S. bulbocastanum*, it would make sense that some of newly identified resistance genes in *S. bulbocastanum* might already have been identified in related species, such as the resistances found in *S. cardiophyllum* and *S. pinnatisectum* (Kuhl et al. 2001).

Allele mining in *Petota* germplasm with primers identifying known *R* genes allows the potato breeder to identify more amenable species for introgression breeding. For example, *S. bulbocastanum* hardly crosses with cultivated potato (Hermesen and Ramanna 1973; Helgeson et al. 1998), and the identification of *Rpi-blb1* alleles in *S. stoloniferum* largely facilitates the introgression breeding of *Rpi-blb1*-like resistance genes for commercial usage (Vleeshouwers et al. 2008; Wang et al. 2008).

Given the high diversity of genes found, some promising *R* genes might not even be present in the germplasm collections. New, extensive collections in hot spots of co-evolution and subsequent evaluations will undoubtedly provide new sources of resistance that can be used in classical and advanced resistance breeding (Haverkort et al. 2009).

MATERIALS AND METHODS

Plant material.

The *Solanum* spp. used were obtained from the CGN. DNA samples of 14 *S. bulbocastanum* accessions originating from the United States potato GenBank, in Sturgeon Bay, WI were supplied by A. del Rio, University of Wisconsin, (referred to as PI accessions in Tables 1 through 3). When available, up to 10 genotypes per accession were used. Individual clones listed in Table 4 were retrieved from the plant material described by Jacobs and associates (2008) for in vivo experiments consisting of detached leaf assays and effector agroinfiltrations.

PCR amplification,

DNA sequencing, and computer analyses.

Specific primers used to amplify fragments of the three *R* genes studied are listed in Supplementary Table S3 and Figure 3. Primers were designed to specifically amplify part of the genes under study and not the known paralogous sequences; for example, not RGA-3 and RGA-4 in the case of *Rpi-blb1* (van der Vossen et al. 2003), the 15 Mi- homologs of *Rpi-blb2* (van der Vossen et al. 2005), and the 24 *Rpi-blb3* homologs known (Lokossou et al. 2009). This requirement considerably reduced the length of the fragment that could be sequenced from each gene: 1,709 bp for *Rpi-blb1*, 715 bp for *Rpi-blb2*, and 618 bp for *Rpi-blb3*. PCR products of randomly selected positive genotypes within an accession were

sequenced directly to confirm their identity and to identify SNP. Sequencing reactions were performed using a dye terminator cycle-sequencing reaction kit (Perkin-Elmer, Pt Biosystem, Warrington, U.K.) and analyzed on an ABI3700 automated sequencer (Applied Biosystem, La Jolla, CA, U.S.A.). Multiple sequence alignments were conducted using DNASTAR Lasergen MegAlign version 8.0.2, permitting the distinction of the SNP compared with the cloned *Rpi-blb* gene sequence (*Rpi-blb1*, GenBank accession number AY426259.1; *Rpi-blb2*, GenBank: DQ122125.1; and *Rpi-blb3*, GenBank: FJ536326.1). Unique sequences are reported in Tables 1 and 2. Nucleotides are numbered from the first nucleotide of the coding sequence of the cloned gene. The synonymous or silent SNP not leading to a change in amino acid were differentiated from the nonsynonymous SNP.

Resistance assay.

Detached leaf assays were used to determine the resistance phenotypes of the tested genotypes with eight *P. infestans* isolates: PIC99177, PIC99189, PIC99183, IPO-C, 90128, and UK7824. Inoculum preparations and inoculations were performed as described by Vleeshouwers and associates (1999). Six days after inoculation, infection symptoms were scored on a scale from 1 to 8, to discriminate from very susceptible until very resistant. Score 1 to 3 meant clear infection, with 1 = heavily sporulating lesion and 3 = ample sporulating lesion, thus characterized as susceptible (S). Scores 4 and 5 were not clearly susceptible or resistant and, therefore, called quantitative (Q). Resistant (R) phenotypes were characterized by score 6, with hypersensitive lesions larger than 10 mm; score 7, lesions between 4 and 10 mm; and score 8, with hypersensitive lesions smaller than 4 mm in diameter.

Effector agroinfiltration on wild species.

The Avr effectors *Avrblb1* (Vleeshouwers et al. 2008) and *Avrblb2* were cloned into vector pK7WG2 and *Pi-Avr2* was cloned into vector pGRAB (Lokossou et al. 2009). The resistance genes *R3a* (Huang et al. 2004) and its cognate effector *Pi-Avr3a* (Bos et al. 2006) were cloned into the vector pGRAB (provided by S. N. Chapman) and used as control for the agrotransformation efficiency. The constructs were transferred into the same *A. tumefaciens* AGL1 carrying pVirG. The different constructs were grown in Luria-Bertani (LB) medium supplemented with chloramphenicol at 75 mg/liter, kanamycin at 50 mg/liter, and tetracycline at 4 mg/liter for *Pi-Avr2*, *Avr3a*, and *R3a*, and with spectinomycin at 100 mg/liter, chloramphenicol at 75 mg/liter, and tetracycline at 4 mg/liter for *Avrblb1* and *Avrblb2*. Inoculum of the first experiment was prepared using the growth of bacteria in LB media (agar at 15 g/liter, bacterial

peptone at 10 g/liter, NaCl at 10 g/liter, and yeast extract at 5 g/liter) until stationary phase and subsequently spun down at 3,600 rpm. Bacteria were resuspended in MMA media (10 mM acetosyringone, 10 mM morpholineethanesulfonic acid, and MS salts at 5 g/liter). In the second experiment, the bacteria were precultured in 3 ml of LB, after which they were transferred to yeast-beef extract medium (beef extract at 5 g/liter, yeast extract at 1 g/liter, sucrose at 5 g/liter, bacterial peptone at 5 g/liter, and 2 mM MgSO₄) and allowed to grow to an optical density at 600 nm (OD₆₀₀) of 0.4 to 2.0; afterward, they were spun down at 3,600 rpm for 10 min and, subsequently, resuspended in MMA medium. Individual constructs were prepared at an OD₆₀₀ of 0.2 and the combination *Pi-Avr3a* /*R3a* at 0.3 were used to infiltrate 3- to 5-week-old wild species plants, reaching their ninth leaf stage (Vleeshouwers and Rietman 2009). Specific recognition of the Avr gene by a putative R gene present induced the hypersensitive cell death response from 5 days post infiltration. Response intensity was scored in percentage of infiltrated area showing cell death: 0 to 20% of dark lesion were characterized by –, 21 to 35% by +, 36 to 65% by ++, and 66 to 100% by +++.

Geographical localization of the accessions.

Geographical origin (latitude and longitude) of the accessions was retrieved from the CGN website and from literature (Rodriguez and Spooner 1997; Hijmans et al. 2007). The desktop application Google Earth was used to visualize the location of the genotypes. Different tags were associated with genotypes containing *Rpi-blb1*, *Rpi-blb2*, *Rpi-blb3*, or their combinations.

We used the internet application SUPRAmap (Janies et al. 2007) in order to associate the different haplotypes with their geographic distribution. The out-group for *Rpi-blb1* and *Rpi-blb3* was assigned by performing a phylogenetic analysis in Mega Software (version 4), using the “minimal evolution” option with standard parameters. The classification described in Tables 1 and 3 was used to illustrate the geographical localization of groups B and C (Table 3) and the M1 clade from Table 1 is highlighted to illustrate the spread of the M1 allele.

ACKNOWLEDGMENTS

We thank D. Janies (Department of Biomedical informatics, Ohio State University, U.S.A.) for his kind help and access to the SUPRAmap website; the Scottish Crop Research Institute (SCRI) and The Sainsbury Laboratory (TSL) for giving us the opportunity to use the effectors *Pi-Avr2* and *Avrblb2*, respectively; N. Champouret (Wageningen UR Plant Breeding) for cloning *Avrblb2* into the expression vector; and P. R. J. Birch, G. Kessel, and F. Govers for providing *Phytophthora* isolates. Funding for this project was provided by the EU (contract no. FOOD-CT-2005-513959 BIOEXPLOIT) for the Bioexploit project and a grant from the Wageningen University Fund.

Fig. 3. Location of *Rpi-blb* gene-specific primers. **A**, *Rpi-blb1*; **B**, *Rpi-blb2*; and **C**, *Rpi-blb3* nucleotide sequences are from the start to the stop codon and drawn to scale. Regions corresponding to the leucine zipper or the coil-coiled domain, the intron, the nucleotide-binding site domain, and the leucine-rich repeat domain are highlighted in gray, white, light gray, and black, respectively. Primer pairs used in this study are 517/1519, Blb1 F/Blb1 R, 1521/518, 1/1', Blb2F/Blb2R, Blb3F/Blb3R, and Blb3F/Blb3Ra.

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AUTHOR-RECOMMENDED INTERNET RESOURCES

The Centre for Genetic Resources, the Netherlands (CGN) website:
www.cgn.wur.nl