Cloning and Characterization of *R3b*; Members of the *R3* Superfamily of Late Blight Resistance Genes Show Sequence and Functional Divergence

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Submitted 30 November 2010. Accepted 26 May 2011.

Massive resistance (*R*) gene stacking is considered to be one of the most promising approaches to provide durable resistance to potato late blight for both conventional and genetically modified breeding strategies. The *R3* complex locus on chromosome XI in potato is an example of natural *R* gene stacking, because it contains two closely linked *R* genes (*R3a* and *R3b*) with distinct resistance specificities to *Phytophthora infestans*. Here, we report about the positional cloning of *R3b*. Both transient and stable transformations of susceptible tobacco and potato plants showed that *R3b* conferred full resistance to incompatible *P. infestans* isolates. *R3b* encodes a coiled-coil nucleotide-binding site leucine-rich repeat protein and exhibits 82% nucleotide identity with *R3a* located in the same *R3* cluster. The *R3b* gene specifically recognizes *Avr3b*, a newly identified avirulence factor from *P. infestans*. *R3b* does not recognize *Avr3a*, the corresponding avirulence gene for *R3a*, showing that, despite their high sequence similarity, *R3b* and *R3a* have clearly distinct recognition specificities. In addition to the *Rpi-mcd1/Rpi-blb3* locus on chromosome IV, the *R3* locus on chromosome XI is the second example of an *R*-gene cluster with multiple genes recognizing different races of *P. infestans*.

Potato (*Solanum tuberosum* L.) is the most important noncereal crop plant and ranks in fourth place in world food production (Reader 2009). This crop is continually threatened by a dozens of pathogens and pests that result in billions of U.S. dollar losses annually. Late blight, caused by the oomycete *Phytophthora infestans*, still remains the most important pathogen in potato-producing regions of the world, causing approximately \$6.7 billion losses annually (Haverkort et al. 2008). Management of this devastating pathogen is challenged by its remarkable speed of adaptation to control strategies such as genetically resistant cultivars by using major resistance (*R*) genes (Fry 2008; McDonald and Linde 2002).

Disease management is currently based on the application of fungicides but it imposes high input costs and results in air, water, and soil pollution. In spite of the application of high amounts of chemicals, late blight is increasingly more difficult to control. Introduction of resistance genes from wild *Solanum* spp. into potato cultivars is considered to be the most promising and environment-safe approach to achieve late blight resistance. However, introgressed resistances that are based on single *R* genes have proven to be not durable. Therefore, an *R*-gene stacking approach is postulated to be required for durability (Jacobsen and Hutten 2006). This can be achieved through marker-assisted breeding or *trans*- or *cis*-genic strategies (Jacobsen and Schouten 2007). The first approach requires accurate mapping positions and robust molecular markers. This can be achieved by cloning the genes responsible for resistance, which is a prerequisite for the second approach, *trans*- or *cis*-genesis. Therefore, efforts from several laboratories worldwide focus on the identification of resistance sources and mapping of the corresponding traits. From the wild species *S. demissum* alone, 11 pathogen race-specific resistance classes have been identified. Several of them were mapped on the short arm of chromosome 11 (Bradshaw et al. 2006; El Kharbotly et al. 1994, 1996), including the *R3* locus (Huang et al. 2004).

The *R3* locus of potato (*S. tuberosum* L.) confers full resistance to avirulent isolates of *P. infestans*. *R3* resides on the short arm of chromosome 11 and segregates in the diploid SH83-92-488 (SH) × RH89-039-16 (RH) population, which was used to construct the ultra-high-density (UHD) amplified fragment length polymorphism (AFLP) potato map (Huang et al. 2004; Isidore et al. 2003; van Os et al. 2006). The *R3* locus is composed of two functional genes, *R3a* and *R3b*, which are 0.4 centimorgans (cM) apart. A recombination event between the two genes allowed their separation, and successive characterization of recombinant plants showed that the two genes have different pathogen isolate-recognition specificities (Huang et al. 2004). Isolate race specificity is a phenomenon common to host-specific resistance and is explained by an arms race that has taken place during co-evolution (Boller and He 2009; Dangl and Jones 2001; Parker and Gilbert 2004). The *R*-gene product directly or indirectly recognizes a pathogen component which is referred to as an effector. This recognition results in an incompatible inter-

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action on plants harboring the resistance gene and, therefore, the cognate effector is also referred to as an avirulence (*Avr*) gene. The effector recognized by the *R3a* gene has been identified and is referred to as *Avr3a* (Armstrong et al. 2005). The *R3a* gene has been isolated via a combination of map-based cloning strategy and resistance gene analog approach (Huang et al. 2005a). It encodes a putative coiled-coil nucleotide-binding site leucine-rich repeat (CC-NBS-LRR) protein and shares 88% nucleotide identity and 83% amino acid similarity with *I-2*, a tomato gene conferring resistance to the soilborne fungus *Fusarium oxysporum* (Simons et al. 1998).

In this study, a map-based cloning strategy was used to clone the *R3b* gene, a paralog of *R3a*. We also found that *Avr3b* was specifically recognized by *R3b* and not by *R3a*. Both isolate spectrum and effector recognition showed that *R3a* and *R3b*

have clearly distinct recognition specificities. Therefore, *R3b* is a new candidate for *R*-gene stacking approaches and the cloning of the gene provides tools for its introduction into new cultivars.

RESULTS

High-resolution genetic mapping of the *R3b* locus.

In the potato UHD map produced by van Os and associates (2006), the *R3* locus was mapped within the Bins SH11B65 to SH11B67 between two cleaved amplified polymorphic sequence (CAPS) markers, cLET5E4 and CT120 (Huang et al. 2004) (Fig. 1). From 1,748 SH×RH progeny, 34 recombinants in the cLET5E4–CT120 interval were found. These recombinants divided the interval into 35 sub-bins (SB), each equivalent to 0.05 cM. Detached leaf assays (DLA) on these recombinants revealed that *R3b* resides in SB28 (Fig. 1). The closest flanking markers were EACAMCAG_152.6 (SB23), GP185 (SB23), and PCAMATA_4 (SB25) at the proximal side and EATCMCAG_15 (SB30), EAACMCGT_139.2 (SB31), and EATCMCAC_3 (SB31) at the distal side. Of the 34 recombinants, 8 had recombinations at the SB23 to SB31 interval. Later, an additional 1,200 SH×RH progeny were screened using cLET5E4 and CT120 for more recombinants in the *R3b* region and three new recombinants were identified at the SB23 to SB31 interval. Consequently, a genetic map of *R3b* with a resolution of 0.03 cM was successfully constructed (Fig. 1).

Physical mapping of the *R3b* locus.

For the physical mapping of *R3b*, two bacterial artificial chromosome (BAC) libraries of the resistant parent SH were constructed with two different restriction enzymes, *Hind*III and *Eco*RI, which were equivalent to a 10- and 33-fold coverage, respectively, of the potato haploid genome. First, the *Hind*III BAC library was screened with the AFLP and CAPS markers in SB23, 25, 30, and 31 (Figs. 1 and 2). Only two BAC clones, SH96A7 (which carries the GP185 allele in *cis* to

Fig. 1. High-resolution genetic map of *R3b*. **A**, High-resolution map of *R3b*. The sub-bins (SB) are numbered from 1 to 35 in a proximal-distal order. On the right, markers are assigned to sub-bins. **B**, Graphical genotypes of 11 SH83-92-488 × RH89-039-16 (SH×RH) progeny that carry cross-over in the *R3b* region. Black and gray indicate the presence and absence, respectively, of the marker allele in *cis* to *R3b*. The transition of black and gray indicates a cross-over.

Fig. 2. Combined physical and genetic maps of the *R3b* region in both SH83-92-488 (SH) haplotypes. Vertical arrows indicate the genetic positions of markers, disease resistance, and figures in between markers indicate the number of recombinants in between the markers. Gray, hatched lines indicate physical positions of markers in the contig. Gray and black rectangles represent bacterial artificial chromosomes (BAC) in *trans* and in *cis* to *R3b*, respectively. T (T7) and S (Sp6) indicate the orientation of BAC derived from the *Hind*III BAC library, while FP and RP indicate the orientation of BAC derived from the *Eco*RI BAC library. Green and blue boxed arrows indicate the location and orientation of *I-2* gene analogs (GA). Red boxed arrows show the position of retrotransposon sequences. Black arrowheads show the position of short coding sequences upstream of *I-2GA*. Yellow boxed arrow shows the position of a putative transposase associated with mutator elements. Hatched arrows indicate the suggested sequential duplications of *I-2GA*.

R3b) and SH61O10 (which carries the EATCMAGC_15 allele in *cis* to *R3b*), were identified. Unfortunately, BAC ends of SH96A7 could not be developed into polymerase chain reaction (PCR) markers. Also, no new BAC was identified using marker SH61O10-T. Therefore, the gap between BAC SH96A7 and SH61O10 could not readily be bridged.

In a previous study, a contig of six BAC (PGEC152I15, PGEC417F14, PGEC481F15, PGEC409I1, PGEC563P22, and PGEC400L14), hereafter referred to as the PGEC152I15-400L14 contig, in the *R7* region was determined to span the *R3b* region in one of the haplotypes in plant introduction (PI) 161729 of *S. demissum* (Dr. Barbara Baker, University of California Berkeley, *personal communication*). This contig was fully sequenced, spanning a physical distance of 488 kb. To bridge the SH96A7–SH61O10 gap in our SH-BAC library, several markers (Table 1) were developed from the PGEC152I15-400L14 contig and mapped in the *R3b* high-resolution genetic map. Successively, several BAC clones from the SH BAC library were identified using these new PGEC152I15-400L14 markers. Two BAC (SH243M3 and SH118E4) are linked in *cis* to *R3b*. SH118E4 end markers were developed and showed that the SH96A7–SH61O10 gap was narrowed but still not closed. Five other overlapping BAC clones (SH70M4, SH159B22, SH51H23, SH27O14, and SH87F20) were determined to be in

trans to *R3b* (Fig. 2) and constituted a new BAC contig referred to as the *r3b* region. Sequence analysis of BAC SH159B22 and SH27O14 revealed the presence of an *I-2* gene analog (GA) cluster (referred to as the SL8 cluster) in the *r3b* region. The SL8 cluster consisted of multiple transposable elements, five complete *I-2*GA, and five partial pseudogenes with homology to *I-2* (Table 2). Although the SH118E4–SH243M3 gap remained in the *R3b* region, sequence information from the *trans* phase was used to produce new *cis* phase markers. Despite many efforts, only one *cis*-phase marker could be developed, showing an apparent lack of synteny between the *r3b* and *R3b* region. CAPS marker SH51H23-T was cosegregating in *cis* with *R3b*.

SL8 primers (Table 1) and the *cis* phase marker SH51H23-T were used to screen the SH library and a new BAC, SH193J21, was identified. SH193J21 largely overlapped with BAC SH118E4 and yielded no new sequence information for marker development. Therefore, BAC SH193J21 and SH159B22 were sequenced, and several sequence-characterized amplified region (SCAR) markers (SH193J21-T4, SH159B22-S, and SH159B22-T4) in *cis* to *R3b* were developed. In addition, SH159B22-T4 and SH193J21-T4 cosegregated with *R3b*. Unfortunately, no new BAC clones in this region were identified using these three new SCAR markers in the *Hind*III BAC library. A new

Table 1. Overview of markers used for mapping *R3b*^a

Marker	Orientation of the primer	Sequence (5'–3')	Annealing temperature (°C)	Restriction enzyme
cLET5E4	F	CCAGGCATGCTCAATTTGGAGT	55	<i>Hha</i> I
	R	TTCCCTGTTTGGACTACTTGTGGA	...	...
CT120	F	CGAGGGGGCGAAGGATT	52	<i>Tsp</i> 509I
	R	CCATGAGATAAACGAGGAACCACT	...	...
SL8D	F	AATTGGAGAGTTCCTACACTTGAG	50	<i>Alu</i> I
	R	AGGGAGGAGGGCAGTGCTGATTC	...	...
SL8E	F	GAATGTTCTCTTTGATAGGCTTGCTC	50	...
	R	GGGCAATCCTCCTTCAGGAAADGA	...	...
GP185	F	CTGGTAATAGTAGTAATGATTCTTCGTC	54	<i>Bst</i> UI
	R	TTGTTCAATGGAGCACTTGC	...	...
542S	F	GTCTGCCCCGTATACCCCTCTTC	52	<i>Apo</i> I/ <i>Mse</i> I
	R	CAGCCGTTGGTGTCTTTGTTCTTG	...	...
141T	F	GATTGATCTAATTGGATGCATTG	...	<i>Ssp</i> I/ <i>Hinf</i> I
	R	TTATTCCGGTGTCTGATATCGC	...	...
656T	F	CAAGTCATGGAATAAACATGG	...	HPYCH41V
	R	AATTGTTCTTTTCTTCAGAATGC	...	...
SH159B22-S	F	CATGCATGTAGGAGGAGGAG	55	<i>Taq</i> I
	R	TAGTTGAATGCTCATTCTCTAG	...	...
SH243M3-S	F	GCTGTGATCATCTTGGAATG	54	...
	R	ATATGTTCTTTCTCCTTGTTAGC	...	...
SH159B22-T4	F	TTGATGGATCCTTTCCCGC	55	SCAR
	R	AATCGTGCGAGCTCACACC	...	...
SH51H23-T	F	TAAGCTTGTCTTCCATAAC	54	<i>Bst</i> UI
	R	TTCCGTCATATAAAGAGATC	...	...
SSH193J21T2-F4	F	GCCCAATCGACATATTATTCACC	62	SCAR
	R	GCTAAATTATTGTTCTTGCAGGTGC	...	...
SH193J21-S2	F2	GATGTTTGCATCGTACTATGG	60	SCAR
	R2	GGTCTAGACAAATGTGGTGTGTG	...	...
EB61A-FP	F	GAAGGAAGCTGTCCAGTGC	60	...
	R	GAAGATGCCGCTACGTCTGAG	...	...
EB61A-RP	F	CAATCGAATATTATCGCTGTG	55	SCAR
	R	ATGGGTTAAGATTGTATGGG	...	...
EB70H-FP	F	CAAGGCACCTGCTATCTCC	54	SCAR
	R	TTGTCTTGCAGGCATTGAG	...	...
EB70H-RP	F	GTGGTACTGTTTATGCATGG	56	a.s.
	R	TTCGTCGAGAAATTTCTCTGTG	...	...
EB18D-FP	F	GAAACTCGTTGAACTCTGCTTGC	60	...
	R	TGTGCCAGAGAATGATGTAGGC	...	...
EB18D-RP	F	AATCTCGAGCTCATGCACG	55	SCAR
	R	TTTCTTGCACCCGTCTTCC	...	...
EB70E-FP	F	GACATTGGGCTAAGACCAAAC	54	a.s.
	R	GTTTGGTCTTAGCCCAATGTC	...	...
EB70E-RP	F	CCATTTTCTTCATTACTAGTCG	55	<i>Alu</i> I
	R	TATACCCACACAAATGTGTATC	...	...

^a F, forward primer; R, reverse primer; SCAR, sequence-characterized amplified region; a.s., allele specific.

BAC library was constructed with restriction enzyme *EcoRI* and screened with these three different SCAR markers. In this new library, several BAC clones (EB18D, EB61A, EB70H, and EB70E) in *cis* to *R3b* were identified, further narrowing the gap (Fig. 2). Based on BAC end sequences, two markers cosegregating in *cis* (EB18D-RP and EB70E-RP) were developed by which two new overlapping BAC clones (EB70HA and EB46B) were identified. Unfortunately, also in the *EcoRI* library, no additional BAC were found that bridged the gap between EB46B and EB70E. To identify *I-2* homologs in the identified BAC clones, *I-2GA* sequences, amplified from SH193J21 using SL8E primers, were used as a probe for Southern blot analysis. *I-2GA* were detected in SH118E4, SH193J21, SH96A7, and EB70E but not in EB18D, EB70H, and EB46B (data not shown). Because of their co-segregation with *R3b*, *I-2GA* in EB70E and SH193J21 were considered to contain candidate genes for *R3b*.

Selection of *R3b* candidate genes.

BAC clones EB70E and SH193J21 were sequenced. Annotation of EB70E and SH193J21 predicted five *I-2GA*, designated as *I-2GA-EB70Ea*, *I-2GA-EB70Eb*, *I-2GA-EB70Ec*=*I-2GA-SH193J21b*, *I-2GA-SH193J21a*, and *I-2GA-SH193J21c* (Fig. 2; Table 2). *I-2GA-SH193J21a* was considered to be a pseudogene because of the presence of several frameshifts. *I-2GA-EB70Eb* and *I-2GA-SH193J21b* contained insertions of retrotransposon sequences; *I-2GA-EB70Ea* and *I-2GA-SH193J21c* contained uninterrupted single-exon open reading frames and were considered the most likely *R3b* candidates. *I-2GA-EB70Ea* and *I-2GA-SH193J21c* were subcloned into binary vector pBINPLUS (van Engelen et al. 1995) using partial digestion of the original BAC with enzyme *Sau3A*. The right subclones were selected using specific PCR and restriction-enzyme fingerprinting and were successively transformed into *Agrobacterium* sp. strains COR308 and AGL0.

Candidates were transiently expressed in *Nicotiana benthamiana* leaves and successively inoculated in DLA with *P. infestans* isolates 89148-9 (*Avr3a* and *Avr3b*) and IPO-0 (*Avr3a* and *avr3b*). Phenotypes were scored at 7 days postinoculation. Only subclone EB70E-76, which contained *I-2GA-EB70Ea* and its up- and downstream (2,150 and 2,140 bp, respectively) sequences, gave a resistant phenotype on the *N. benthamiana*

leaves inoculated with the *R3b*-incompatible late blight isolate 89148-9 (data not shown). The leaves expressing the other subclones, including the subclone SH193J21-321 that contained *I-2GA-SH193J21c*, showed large sporulating lesions after inoculation with 89148-9. As expected, full susceptibility was observed with all constructs, including EB70E-76, when inoculated with the *R3b*-compatible isolate IPO-0.

Complementation analysis in stable transgenics.

The genes *I-2GA-EB70Ea* and *I-2GA-SH193J21c*, together with 2- to 3-kb up- and downstream sequences, were introduced into susceptible ‘Desiree’ plants through *Agrobacterium*-mediated transformation. At least 10 in vitro plantlets of each primary transformant were tested for resistance to *P. infestans* isolates 89148-9 and IPO-0 in duplicate experiments. Desiree and RH were included as susceptible controls, and SH and SW8563-618 (*R3b* recombinant) were included as resistant controls. Upon analysis of the primary transformants, only subclone EB70E-76, containing *I-2GA-EB70Ea*, was able to specifically complement the susceptible phenotype (Fig. 3A), which was consistent with the transient complementation results in *N. benthamiana* described in the previous section. All primary transformants of this subclone exhibited *R3b*-specific resistance to isolate 89148-9 and susceptibility to IPO-0 (Fig. 3A). The results were confirmed using a DLA in duplicate tests (Fig. 3B). Because subclone EB70E-76 contained only one gene, we conclude that *I-2GA-EB70Ea* is the *R3b* gene.

R3b specifically recognizes *Avr3b*.

In parallel studies, *Avr* genes matching specific *R* genes are being identified by high-throughput effector genomics (Oh et al. 2009; Vleeshouwers et al. 2008). Effector candidates carrying a signal peptide and RXLR motif were mined from the recently sequenced *P. infestans* isolate T30-4 (Haas et al. 2009; Haverkort et al. 2008), which is avirulent to *R3b* plants (Huang et al. 2004). Effectors were expressed through *Agrobacterium tumefaciens* (Rietman 2011) and co-infiltrated with *R3b* in *N. benthamiana* leaves. One clone, PITG_18215, was specifically recognized by *R3b*, as was evident from the induced hypersensitive response (Fig. 4A) (Rietman 2011). *R3b* did not recognize *Avr3a*, and neither did *R3a* recognize *Avr3b* (Fig. 4B). These results further functionally confirm the iden-

Table 2. Overview of sequences from different haplotypes in the *R3b* region^a

Haplotype	BAC (accession number)	Complete <i>I-2GA</i> (position)	Partial <i>I-2GA</i> and pseudogenes (position)
PI161729- <i>r3b</i>	PGEC152I5 (AC136471)	...	...
	PGEC417F14 (AC149289)	...	...
	PGEC481F15 (AC149488)	<i>Sd</i> -481F15-a (29270-33214) <i>Sd</i> -481F15-b (41747-45655)	<i>Sd</i> -481F15-c (78315-82084) ...
	PGEC409I1 (AC142210)	...	...
	PGEC563P22 (AC150163)	...	<i>Sd</i> -563P22-a (5170-10028) <i>Sd</i> -563P22-b (13560-16171)
	...	...	...
	PGEC400L14 (AC149302)	<i>Sd</i> -400L14 (13560-16171)	...
Haplotype	BAC (accession number)	Complete <i>I-2GA</i> (accession number)	Partial <i>I-2GA</i> and pseudogenes (accession number)
SH- <i>r3b</i>	SH159B22	SH159B22b (EF638450) SH159B22c (EF638451)	SH159B22a (EF638459)
	SH27O14	SH27O14e (EF638455) SH27O14f (EF638456) SH27O14b (EF638454) SH27O14g (EF638457)	SH27O14a (EF638461) SH27O14c (EF638462) SH27O14d (EF638463)
	SH96A7	SH96A7a (EF638458) SH96A7b (EF638464)	SH96A7c (EF638465)
	SH193J21 (JF900492)	SH193J21c (EF638453)	SH193J21a (EF638460) SH193J21b = EB70Ec (EF638452)
	EB70E (JF900492)	EB70Ea = <i>R3b</i> (HQ437670)	EB70Eb (HQ437669)

^a Bacterial artificial chromosome (BAC) clones and *I-2* gene analogs (GA).

tity of *R3b*. Also, it shows that *R3b* and *R3a* have clearly distinct recognition specificities.

***R3b* gene structure and predicted amino acid sequence.**

The *R3b* gene consists of one exon containing a single open reading frame of 3,855 nucleotides. The predicted *R3b* transcript encodes a polypeptide of 1,284 amino acids. The structure of the *R3b* gene is similar to *R3a*, encoding a putative CC-NBS-LRR protein. *R3b* and *R3a* exhibit 82% identity at the nucleotide level and 73% similarity at the amino acid level. The N-terminal half of the *R3b* protein contains three potential CC domains and seven conserved NB-ARC subdomains (Fig. 5). *R3b* and *R3a* proteins are quite conserved in the CC domain (79% amino acid [aa] identity) and NBS domain (75% aa identity), especially at the motifs that define the domain (86% aa identity). The C terminus of *R3b*, referred to as LRR in Figure 4, shares 67% aa identity with *R3a*.

The C-terminal half of *R3b* comprises a series of 28 irregular LRR that can be aligned according to the consensus sequence xxLxLxx observed in other cytoplasmic R proteins. Although most of the LRR in *R3a* and *R3b* can be aligned to each other, obvious differences were observed between them. The *R3a* gene encodes 29 LRR and the *R3b* gene only 28 (Fig. 5, line 30). In addition, both genes have two unique LRR (Fig. 5, lines 14/15 and 26/27). In the LRR domain, only 45% (81/182) of the putative solvent-exposed residues (x in the frame xxLxLxx) were conserved between *R3a* and *R3b*. This hypervariability fits the hypothesis that the LRR region of R proteins may define recognition specificity. Among the known *P. infestans* R genes, *R3b* shares highest identity with *R3a* and bears limited similarity (50% identity in nucleotides and 34% in amino acids) to *RB/Rpi-blb1* (Song et al. 2003; van der Vossen et al. 2003). All other known late blight R proteins—R1 (Ballvora et al. 2002), Rpi-blb2 (van der Vossen et al.

Fig. 3. Functional complementation of *Phytophthora infestans* susceptibility in 'Desiree'. **A**, In vitro inoculation of the primary transformants of Desiree. Nontransgenic Desiree plants are susceptible to both isolates. Transgenic Desiree plants containing *I-2* gene analog (GA)-EB70Ea are resistant to the isolate 89148-9. **B**, Detached leaf assay with greenhouse-grown transgenic Desiree plants. Desiree and Desiree stably transformed with *I-2GA-EB70Ea* were inoculated on the right side of the leaf with isolate 89148-09 and on the left side with isolate Dinteloord. The picture taken 6 days postinoculation is a representative photograph out of 50 individual transformed Desiree clones.

2005), Rpi-blb3 (Lokossou et al. 2009), and Rpi-vnt1 (Pel et al. 2009)—showed less than 30% identity.

A phylogenetic analysis of the I-2 protein analogs described in this study and available homologous sequences from GenBank was performed. The Rpi-blb1 family of R proteins was used as an outgroup. Two distinct, bootstrap-supported clades of I-2 protein analogs could be distinguished (Fig. 6). The upper clade contained I-2 and the R3a protein and the lower clade contained the R3b protein. This fitted the assumption that R3a and R3b, both located in the R3 gene cluster, are clearly distinct, and together, they define a new R-gene superfamily.

DISCUSSION

Location of R3b in R3 supercluster.

R3b was, like R3a, mapped to the distal part of the short arm of potato chromosome 11 in the diploid segregating population of SH×RH (Huang et al. 2004). The R3b gene is located distal to marker GP185, whereas R3a was located proximal to GP185. Like many other R genes, R3b resides in a hot spot for resistance to various pathogens. To date, the tomato I-2 gene encoding resistance to *F. oxysporum* and the R3a gene conferring resistance to *P. infestans* were cloned from this region. In this study, we have shown that the R3b gene encodes a CC-NBS-LRR protein and is a paralog of R3a (Huang et al. 2005a). R3a and R3b are located in the same R3 supercluster, separated by 0.4 cM. The physical distance in *cis* phase could not be determined because of a remaining gap in the BAC contig. In the *trans* phase, the *r3b* and *r3a* regions are estimated to be 550 kb apart. In addition to their physical distance, phylogenetic and genetic analysis showed that R3a and R3b regions are clearly distinct. The R3b gene and 11 homologs form a clade in the tree (Fig. 6) that is clearly different from the clade that contains R3a and I-2. Interestingly, all genes in the R3b clade are located distal to marker GP185.

Evolution of the R3b subcluster.

The R3b gene is most similar to I-2GA-SH193J21c, exhibiting 97.7% nucleotide identity. Comparison of the R3b and I-2GA-SH193J21c sequences showed that these two genes may have undergone two sequence exchanges because of an unequal distribution of single-nucleotide polymorphisms (SNP). The first exchange tract (1,403 bp, from +7 to +1,409 of the R3b gene) showed only three SNP between the two genes, and the second exchange tract (+1,907 to +2,598) was identical in the two genes. Therefore, the R3b gene seems to have an evolutionary pattern of type I R genes (Kuang et al. 2004). I-2GA-EB70Eb and I-2GA-SH193J21b, which are physically separating R3b from its closest homolog, I-2GA-SH193J21c, are highly similar and contain insertions of retrotransposons at identical positions in their coding regions. We hypothesize that I-2GA-SH193J21a and I-2GA-SH193J21c duplicated to form R3b and I-2GA-EB70Eb. Successively, two retrotransposons (10.8 kb total) were inserted into I-2GA-EB70Eb. I-2GA-EB70Eb was then duplicated to form I-2GA-SH193J21b, and the latter product lost, successively, 8 kb of the retrotransposon sequence. These proposed sequential duplication events have been indicated in Figure 2 by hatched arrows.

Lack of synteny between haplotypes in R3b region.

The comparison of the R3b region in three haplotypes (SH *cis*, SH *trans*, and *S. demissum*) showed that this region varies dramatically. Six I-2GA were present in the 488-kb sequence at the R7 region in *S. demissum* (contig PGEC15215-400L14; accession PI161729), of which three are complete genes (Table 2). In this study, 10 I-2GA were identified from BAC SH159B22 and SH27O14 in the *r3b* region, of which 5 were full-length genes. The R3b region in SH (BAC clones SH96A7, EB70E, and SH193J21) harbors eight I-2GA, of which four are full-length genes (Table 2). SH96A7 is allelic to BAC542 in the PGEC15215-400L14 contig from *S. demissum*.

Fig. 4. R3b and R3a effector recognition specificity. *Nicotiana benthamiana* leaves were co-agroinfiltrated with avirulence (Avr) effectors and the potato resistance gene A, R3b and B, R3a. The following constructs were co-infiltrated with the resistance (R) genes: 1, R + pMDC32 empty plasmid (negative control); 2, R + Avr3aKI; 3, R + AVR2; 4, R3a + Avr3aKI (positive control); 5–7, R + PITG_18215. Co-infiltration was accomplished using a mixture of equal amounts of *Agrobacterium* strains. All bacterial densities were set to an optical density at 600 nm (OD₆₀₀) of 0.2 for spots 1–5. Spots 6 and 7 were infiltrated at OD₆₀₀ = 0.5 and 1.0, respectively. Photographs were taken 4 days postinfiltration.

sum, both harboring the marker GP185 and three *I*-2GA. More distally, microsynteny was sparse in different haplotypes, and no allelic relationship could be determined for the genes in this region, similar to the *R1* locus in *S. demissum* (Kuang et al.

2005) and the *RPP5* locus (Noel et al. 1999) in *Arabidopsis thaliana*. This apparent lack of synteny between haplotypes severely delayed the physical map construction of the *R3b* region, because the markers located in the *r3b* region (except for marker 51T) could not be used to find *cis* phase BAC in the *R3b* region.

Functional diversification between *R3a* and *R3b*.

In potato, most of the *R* genes belong to complex *R*-gene clusters with tightly linked paralogs (Gebhardt and Valkonen 2001). Large arrays of similar sequences allow for equal or unequal recombination events, resulting in the formation of new gene clusters (Hulbert et al. 2001). However, the evolutionary fate of new members in complex resistance loci may largely depend on the interaction between the plant and its pathogens. In gene-for-gene interactions, plant *R* proteins recognize the pathogen *Avr* or effector proteins either through direct *R*–*Avr* protein–protein interaction or indirectly through detection of modifications of host proteins targeted by effectors, the so-called guard proteins in the guard model (Jones and Dangl 2006; McDowell and Simon 2006). Host–pathogen co-evolution will lead to hosts evolving new specificities in their *R* proteins to identify the corresponding *Avr* factors as well as pathogens altering their *Avr* factors to avoid *R*-mediated recognition (Parker and Gilbert 2004).

Most *R*-gene clusters contain only one *R* gene with a described function. Only a few *R*-gene clusters comprise more than one described functional *R* gene (Botella et al. 1998; Dixon et al. 1996; Rouppe et al. 1999; Takken et al. 1999). In addition to the *Rpi-mcd1/Rpi-blb3* locus on chromosome 4, the *R3* locus is the second example of an *R*-gene cluster with multiple genes recognizing different races of *P. infestans* (Lokossou 2010).

Reported mechanisms resulting in conversion of *Avr* to *avr* include nucleotide substitutions, insertions, and deletions. The *R* genes corresponding to the *Avr* genes may also be mutated through point mutations, insertions, and deletions to change into nonresistance genes. In this article, the *R3b* gene and *I*-2GA-*SH193J21c* have an identity of 97.7% but *I*-2GA-*SH193J21c* showed no resistance function with the late blight isolates tested.

R3a was triggered in the host cytoplasm by the *Avr3a* gene (Armstrong et al. 2005) to exhibit hypersensitive cell death. Because of the high level of sequence conservation between *R3a* and *R3b*, and because *Avr3b* encodes an RXLR protein, which is predicted to be sequestered into the host cytoplasm, it can be postulated that *R3b* has a resistance mechanism similar to *R3a*. Its recognition specificity, however, is clearly different because *R3b* does not recognize *Avr3a* (Fig. 4A) and *R3a* does not recognize *Avr3b* (Fig. 4B). This is supported by studies with specific isolates for *R3a* and *R3b* by Huang and associates (2005a). The low extent of homology in the LRR domains of *R3a* and *R3b* compared with the CC and NBS domains suggests that the LRR domain may be involved in pathogen recognition, as is the case in other *R* proteins (Burch-Smith et al. 2007; Jones and Dangl 2006; Lokossou 2010).

Fig. 5. Primary structure and alignment of the *R3a* and *R3b* resistance proteins. Amino acid residues of *R3a* and *R3b* that are identical are shown in normal script. *R3a*- and *R3b*-specific residues are shown in bold at top and bottom lines, respectively. Dashes indicate gaps to fit the alignment. The predicted coiled coils (Lupas et al. 1991) in the coiled-coil domains are underlined. The conserved motifs of the nucleotide-binding site domain are overlined. The consensus xxLxLxx of each leucine-rich repeat is highlighted with a gray background.

Of the 11 *S. demissum* *R* genes, *R3* (including *R3a* and *R3b*), *R6*, *R7*, *R10*, and *R11* have been mapped on chromosome 11 (Bradshaw et al. 2006; El-Kharbotly et al. 1996; Huang et al. 2004). *R5*, *R8*, and *R9* also have been shown to contain the *R3* gene locus (Huang et al. 2005a). It will be interesting to know why so many *R* genes locate on chromosome 11, most likely in the *R3* supercluster. Additional research investigating functional diversity of the respective *S. demissum* *R* genes might be greatly facilitated by the identification of their cognate Avr component.

Implications for plant breeding.

Though many *R* genes were identified in different species and several *R* genes have successfully been transferred to cultivated potato (Golas et al. 2010; Huang et al. 2005a; Pel et al. 2009; Sliwka et al. 2010; van der Vossen et al. 2003), it is generally assumed that individual *R* genes are easily overcome by the variable pathogen *P. infestans*. Stacking of *R* genes with broad-spectrum major resistance genes could be a desirable solution (Lokossou 2010; Rauscher et al. 2010; Stewart et al. 2003; Tan et al. 2010). However, selection for functional stacking of *R* genes in a susceptible cultivar is highly dependent on the availability of the corresponding Avr genes, as has been shown in triple *R*-gene transformants of Desiree by Zhu and associates (2010). This combined availability is fortunately the case with more and more cloned *R* genes. Cloning of both *R3b* and *Avr3b* will provide great help to characterize the *R3* gene cluster in *Solanum* spp. and will promote additional *R*-gene cloning in this region. Newly cloned *R* genes from the *R3* gene cluster will be deployed as stacked cisgenic *R* genes for the improvement of existing potato cultivars using marker-free genetically modified organism techniques (Jacobsen and Schouten 2007).

MATERIALS AND METHODS

Plant material.

The F1 population of SH×RH that segregates for both *R3a* and *R3b* was used for genetic mapping (Huang et al. 2004). The susceptible potato clone Desiree was used for genetic transformation.

P. infestans isolates and inoculation.

P. infestans isolates 89148-09 (Avr3a and Avr3b), IPO-0 (Avr3a and avr3b), and Dinteloord (Avr3b) were inoculated on transgenic in vitro plantlets, on *N. benthamiana* plants, or on leaves of transgenic, greenhouse-grown plants in DLA (Huang et al. 2004).

Genetic mapping.

In total, 3,000 SH×RH progeny were screened for recombinants in the *R3* region using the flanking markers cLET5E4 and CT120 (Table 1). In order to get the recombinants of the *R3b* region, the selected recombinants were screened further using flanking markers GP185 and 656T (Table 1). The selected *R3b* region recombinants were used to determine the position of the *R3b* AFLP markers (Fig. 1). DNA isolation and analysis of molecular markers were as described by Huang and associates (2004).

In the 10,000-marker UHD map, “Bin” instead of centimorgan as the genetic distance unit has been introduced. Bin is defined as a genetic interval flanked by a recombination event on either side (van Os et al. 2006). The population size of the UHD map was 130; thus, a Bin is roughly equivalent to 0.8 cM.

Physical mapping.

Two BAC libraries of the resistant parent SH were constructed with different restriction enzymes *Hind*III and *Eco*RI

according to the previously described protocol (Roupe van der Voort et al. 1999). The *Hind*III BAC library (10× coverage of SH genome) was stored in 255 384-well microtiter plates and their BAC clones were named with prefix SH. The

Fig. 6. Phylogenetic analysis of R3 protein superfamily. R3b homologs were derived from various sources as described in the text. *Sd*- sequences were derived from *Solanum demissum* contig PGEC152115-400L14 and additional sources (10I24a = EF613516; 10I24c = EF613518; 10I24d = EF613519; 568A14g = EF613544; 591C22a, d, e = AC151801). R3a, I-2 gene analog (GA)-SH194-2, and I-2GA-SH23 sequences were derived from bacterial artificial chromosomes SH83-92-488, SH194C17, and SH23G23, respectively (Huang et al. 2005a). I-2 like sequences from tomato were I-2 (AF118127), I2C-1 (AF004878), I2C-2 (AF004879), and I2C-5 (AF408704). Other SH sequences were identified in this study: SH159B22-b = EF638450; SH159B22-c = EF638451; SH27O14e = EF638455; SH27O14f = EF638456; SH27O14b = EF638454; SH96A7a = EF638458; SH96A7b = EF638464; SH193J21c = EF638453; EB70Ea = R3b = HQ437670; Rpi-blb1 and Rpi-sto1 = EU884421 and Rpi-pta1 = EU884422 (Vleeshouwers et al. 2008); RB-ver = EF202329 (Liu and Halterman 2006); and Rpi-bt1 = FJ188415 (Oosumi et al. 2009). Alignments were performed according to the Clustal W algorithm and the closest related gene family, founded by Rpi-blb1, was used as an outgroup. Successively, a bootstrapped distance tree was calculated using 1,000 jumbles and seed = 111 using Megalign from the DNASTar software package.

EcoRI BAC library (33× coverage of SH genome) was stored in 80 super pools, each superpool contained 4,000 to 5,000 clones, and their BAC clones were named with prefix EB.

Screening of the *HindIII* BAC library with *R3b*-region markers was performed as described previously (Huang et al. 2005a). Screening of the *EcoRI* BAC library was performed as follows. First, positive superpools (1 superpool = 4,000 to 5,000 clones) were identified; second, pools (1 pool = 400 to 500 clones) were made from those positive superpools; third, positive pools were identified; fourth, subpools (1 pool = 40 to 50 clones) were made from those positive pools; fifth, positive subpools were identified; sixth, single positive clones were identified using colony PCR. Plasmid DNA was isolated using the standard alkaline-lysis protocol from superpooled, pooled, and subpooled bacteria to produce BAC DNA pools (Roupe van der Voort et al. 1999).

Plasmids of single BAC were purified using MIDI-PREP columns (Qiagen, Hamburg, Germany). Sequences generated through BAC end sequencing with SP6, T7, FP, or RP primers were employed to design PCR primers to develop BAC end markers. The BAC end markers were named according to the BAC or pool number followed by S (SP6), T (T7), FP, or RP (Table 1). In view of the repetitive nature of the *R3* complex locus, the overlap of two BAC was verified by i) *HindIII* restriction mapping, ii) nonselective AFLP fingerprinting using *HindIII* and *MseI/TaqI* (Simons et al. 1998), and iii) analysis of BAC end markers through melting temperature gradient PCR restriction with 24 frequent cutters and sequencing of the PCR products.

DNA sequencing and analysis.

The BAC clones SH27O14, SH159B22, SH96A7, SH193J21, and EB70E (Fig. 2; Table 2) were sequenced by using a shotgun sequencing strategy. Positions of putative genes were predicted using GENSCAN (Burge and Karlin 1997) and GENEMARK (Lukashin and Borodovsky 1998). The alignments of multiple sequences were conducted by using CLUSTALW (Thompson et al. 1997).

Subcloning and transformation of I-2GA.

Candidate I-2GA were subcloned into the binary vector pBINPLUS (van Engelen et al. 1995) from the BAC clone EB70E and SH193J21 as described previously (van der Vossen et al. 2003). Binary plasmids harboring the candidate genes were transformed to *Agrobacterium tumefaciens* AGL0 (Lazo et al. 1991) and COR308 for transient complementation and introduced into Desiree according to standard protocols (Visser et al. 1991).

Agroinfiltrations.

Agroinfiltrations for transient expression of selected genes in *N. benthamiana* followed established protocols (van der Hoorn et al. 2000). For successive *Pi* inoculation, *R*-gene constructs were transformed into *A. tumefaciens* COR308. Bacteria were suspended in infiltration medium to an optical density at 600 nm (OD₆₀₀) of 0.2 and infiltrated into the leaves of 4-week-old *N. benthamiana* plants using a 2-ml syringe. Two days later, the leaves were collected and subjected to DLA. For *R* gene/effector co-expression studies, *A. tumefaciens* AGL1+VirG harbored the effector constructs and the *R*-gene constructs were in COR308. Bacteria were suspended in infiltration medium to OD₆₀₀ = 0.2, mixed in a 1:1 ratio, and infiltrated into the leaves of 6-week-old *N. benthamiana* plants.

Effector analysis.

In a collaborative effort with the Sainsbury lab and Scottish Crop Research Institute, effectors were identified that were

transcriptionally upregulated at 2 to 3 days postinoculation (Haas et al 2009) (Rietman 2011; a detailed description of the compilation of the effector collection will be published elsewhere). Of these, 150 effectors were selected and coding sequences were synthesized by Genscript Inc. (Piscataway, NJ, U.S.A.) in between AttL1 and AttL2 gateway recombination sites and cloned into pUC57. Sequences coding for putative secretion signal peptides were excluded and replaced with an ATG codon preceded by two nucleotides that would allow in-frame N-terminal fusions for other applications. This effector collection was subcloned into pMDC32 using LR clonase (Invitrogen, San Diego, CA, U.S.A.) and transformed into *A. tumefaciens* AGL1+VirG. Construct EB70E-76, containing the *R3b* gene, was used for co-agroinfiltration with individual members of this effector set. Upon recognition of the encoded effector by the *R* gene, a hypersensitive response can be observed between 3 and 5 days postinoculation. As a control, we used *R3a* and *Avr3a-KI* (Armstrong et al. 2005).

ACKNOWLEDGMENTS

We acknowledge the kind support of the National Natural Science Foundation of China (30971995 and 36171319) and Ministry of Science and Technology of China (2007DFB30080). G. Li, E. van der Vossen, and J. Vossen were supported by the DuRPh program granted by the Dutch ministry of Agriculture. We thank Z. Zhang (Chinese Academy of Agricultural Sciences) for bioinformatics analysis and P. Birch (Scottish Crop Research Institute, U.K.) and S. Kamoun (Sainsbury Lab, U.K.) for joint efforts in compiling a genome-wide set of RXLR effectors from *P. infestans*.

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The citation for Rietman 2011 has been added to the online version on September 16, 2011.