Exploiting Knowledge of *R/Avr* Genes to Rapidly Clone a New LZ-NBS-LRR Family of Late Blight Resistance Genes from Potato Linkage Group IV

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In addition to the resistance to *Phytophthora infestans* (*Rpi*) genes *Rpi-blb1* and *Rpi-blb2*, *Solanum bulbocastanum* appears to harbor *Rpi-blb3* located at a major late blight resistance locus on LG IV, which also harbors *Rpi-abpt*, *R2*, *R2-like*, and *Rpi-mcd1* in other *Solanum* spp. Here, we report the cloning and functional analyses of four *Rpi* genes, using a map-based cloning approach, allele-mining strategy, Gateway technology, and transient complementation assays in *Nicotiana benthamiana*. *Rpi-blb3*, *Rpi-abpt*, *R2*, and *R2-like* contain all signature sequences characteristic of leucine zipper nucleotide binding site leucine-rich repeat (LZ-NBS-LRR) proteins, and share amino-acid sequences 34.9% similar to RPP13 from *Arabidopsis thaliana*. The LRR domains of all four *Rpi* proteins are highly homologous whereas LZ and NBS domains are more polymorphic, those of *R2* being the most divergent. Clear blocks of sequence affiliation between the four functional resistance proteins and those encoded by additional *Rpi-blb3* gene homologs suggest exchange of LZ, NBS, and LRR domains, underlining the modular nature of these proteins. All four *Rpi* genes recognize the recently identified RXLR effector PiAVR2.

The potato (*Solanum tuberosum*) ranks as the world's third most important food crop after wheat and rice. Compared with these other staple crops, a greater proportion of the potato crop is edible and a higher yield per hectare is achieved, which, combined with its high nutritional value, underlines its importance and explains ongoing increases in global potato production. The most significant threat to potato production worldwide is the late blight disease, caused by the oomycete pathogen *Phytophthora infestans*, which destroys leaves, stems, and

tubers. Current disease control relies on multiple (up to 20) fungicide applications per season (Hanson et al. 2007) and costs associated with chemical control amount to €5 billion globally per year (Whisson et al. 2001). Such pesticide inputs are both environmentally and economically undesirable and have a negative impact on the sustainability of potato production. Host resistance is a more environmentally benign means of restricting late blight infection but the success of this management practices hinges on effective and durable host resistance and an understanding of the pathogen population.

Early in the 20th century, the potential of the wild Mexican hexaploid species *S. demissum* was recognized for conferring extreme levels of late blight resistance, and hybrids with *S. tuberosum* were generated. Subsequently, 11 major dominant resistance (*R*) genes were identified and introgressed into potato cultivars (Malcolmson 1969; Malcolmson and Black 1966; Müller and Black 1952). However, deployment of these single genes did not provide durable resistance due to the rapidly changing populations of *P. infestans*. As an alternative approach, breeders started to select for quantitative “field” resistance using races of *P. infestans* that were compatible with the *R* genes present in their breeding material (Black 1970; Toxopeus 1964). Despite considerable progress in genetic analysis of quantitative resistance to late blight using molecular markers (Gebhardt and Valkonen 2001; Simko 2002), under long day conditions, breeders have achieved little progress, the major drawbacks being tetrasomic inheritance of potato and the strong linkage between foliage resistance and late foliage maturity (Bradshaw et al. 2004; Oberhagemann et al. 1999; Visker et al. 2003). Therefore, it is anticipated that breeding for late blight resistance in potato, aimed at substantially contributing to disease management, requires the deployment of major resistance to *P. infestans* (*Rpi*) genes. Recent germplasm screens are revealing a wealth of *Rpi* genes in wild *Solanum* spp. and the challenge now is how to select, judiciously combine, and deploy sets of *Rpi* genes that can confer durable late blight resistance in modern potato cultivars.

Key to durable exploitation of *Rpi* genes is also a better understanding of their interactions with effectors from *P. infestans*. All microbes trigger immune responses in plants via host receptor-mediated recognition of pathogen-associated molecular patterns (PAMPs). Such PAMP-triggered immunity (PTI) can be suppressed by secretion of effectors which act either outside

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or inside host cells and reprogram host metabolism to promote effector-triggered susceptibility (ETS). When a successful pathogen has suppressed PTI, the products of plant *R* genes, as a second “layer” of defense, can directly or indirectly detect effectors (termed avirulence [AVR] proteins). *R* proteins subsequently activate resistance responses (effector-triggered immunity [ETI]), often including programmed cell death during the hypersensitive response (HR) (Jones and Dangl 2006). The ability of a pathogen to evade recognition in the *R*-gene-for-Avr-gene concept (Flor 1971) can be explained by an evolutionary “arms race” (Bergelson et al. 2001; Holub 2001). The generation, through mutation, and selection of AVR alleles that evade recognition is matched by strong selection pressure for *R* gene alleles that retain recognition. This is particularly evident in the ATR1-RPP1 and ATR13-RPP13 interaction in the *Hyaloperonospora arabidopsidis* (formerly *H. parasitica*)–*Arabidopsis* pathosystem, where considerable allelic diversity has been revealed by studying natural host and pathogen populations (Allen et al. 2008; Rehmany et al. 2005). In naturally co-evolving *P. infestans*–potato populations, similar high levels of allelic diversity are expected to be observed. So far, studies of the PiAVR3a–R3a interaction reveal diversifying selection for both genes (Armstrong et al. 2005; Huang et al. 2005). Recently, several oomycete AVR genes were identified (Allen et al. 2004; Armstrong et al. 2005; Rehmany et al. 2005; Shan et al. 2004; Vleeshouwers et al. 2008). Although each of the AVR proteins is distinct at the level of primary sequence similarity, they all share a secretion signal peptide followed by the motif RXLR and an acidic region, often ending in the sequence EER (Birch et al. 2008a and b; Whisson et al. 2007). The RXLR-EER region is required for translocation of PiAVR3a, following its secretion from *P. infestans* haustoria, to the inside of host plant cells (Whisson et al. 2007). Translocation of RXLR-containing proteins inside the plant cell is consistent with their recognition by host nucleotide binding site leucine-rich repeat (NBS-LRR) resistance proteins (Birch et al. 2006). Because all oomycete AVR proteins identified to date have the RXLR motif, it provides a signature to identify AVR candidates from the *P. infestans* genome. Future knowledge of RXLR effectors diversity and their survey in *P. infestans* population could predict the lifespan of *Rpi* genes in specific areas.

Conventional introgression of resistance often involves interspecific crosses and repeated backcrosses with cultivated potato, which is a very slow and inefficient process. Currently, *Rpi* genes of natural origin, so-called cisgenes (Jacobsen and Schouten 2007; Schouten and Jacobsen 2008), can be introduced using marker-free transformation systems leading to cisgenic plants with only the genes of interest and without linkage drag of other genes from the wild species or from antibiotic resistance genes normally present at the T-DNA (de Vetten et al. 2003; Schaart et al. 2004; Yu et al. 2006). To date, six *Rpi* genes have been reported: *R1* and *R3a* from *S. demissum* (Ballvora et al. 2002; Huang et al. 2005), *RB/Rpi-blb1* and *Rpi-blb2* from *S. bulbocastanum* (Song et al. 2003; van der Vossen et al. 2003, 2005), and *Rpi-sto1* and *Rpi-ptal* from *S. stoloniferum* and *S. papita*, respectively (Vleeshouwers et al. 2008). All belong to the NBS-LRR class of plant *R* proteins, which are thought to be cytoplasmic (van der Biezen and Jones 1998). Various loci are reported to contain *Rpi* genes on linkage group (LG) VII (Kuhl and Hanneman 2001), LG IX (Sliwka et al. 2006; Smilde and Brigneti 2005), LG X (Ewing and Simko 2000; Rauscher et al. 2006), and LG XI (Bradshaw et al. 2006; Huang et al. 2004, 2005).

Here, we describe the cloning and functional characterization of *Rpi-blb3*, *Rpi-abpt*, *R2*, and *R2-like* from the major late blight resistance locus on LG IV of potato (Li et al. 1998; Park

et al. 2005a, b, and c). Upon map-based cloning of *Rpi-blb3*, an efficient candidate gene allele-mining approach in combination with transient complementation in the heterologous *Nicotiana benthamiana* “model” system led to the rapid cloning of the other three genes. All four *Rpi* genes code for proteins of the NBS-LRR class of *R* proteins. Interestingly, with respect to known functional *R* proteins, they share the highest similarity (34.9% amino acids) with RPP13 from *Arabidopsis thaliana* (Bittner-Eddy et al. 2000), which confers resistance to the oomycete *H. arabidopsidis*. Despite apparent differences between the four functional *R* proteins, all four induce an HR upon coexpression with the *P. infestans*-derived RxLR effector PiAVR2 (E. Gilroy, *personal communication*) in *N. benthamiana* leaves, indicating that they recognize the same effector protein.

RESULTS

Cloning of *Rpi-blb3* and *Rpi-abpt*.

In order to clone *Rpi-blb3* and *Rpi-abpt*, two bacterial artificial chromosome (BAC) libraries were constructed using DNA derived from the resistant clones Blb99-256-3 and 707TG11-1, respectively. Approximately 74,000 clones with an average insert size of 85 kb, corresponding to eight genome equivalents, were obtained for each library. These libraries were screened initially with sequence-characterized amplified region marker Th21, which cosegregated with resistance in mapping populations of 1,396 and 1,383 F1 progeny plants (Park et al. 2005a). In this way, BAC clones Blb22 and TG9, respectively, were identified (Fig. 1A and 1B). By sequencing the ends of these two BACs, new markers were developed which were used to define the genetic intervals of the *R* loci more precisely and to rescreen the BAC libraries to identify clones that overlapped with the initial BAC clones. In this way, the *Rpi-blb3* locus was delimited to a 0.1-centimorgan (cM) interval (EAAC/MAAC_99- Blb25-T; 2/1,396 recombinants) that is physically spanned by the two partially overlapping BAC clones Blb22 and Blb25 (Fig. 1A). In the case of the *Rpi-abpt* locus, the partially overlapping BAC clones TG9 and TG77 were identified. One end of the contig cosegregated with resistance (TG77-S) while the other mapped 0.1 cM proximal to *Rpi-abpt* (TG9-S) (Fig. 1B).

To gain insight into the molecular structure of the *R* loci under study, BAC clones Blb22 and TG9 were sequenced to 6x coverage. This revealed that clone Blb22 did not contain any *R* gene homologues (RGH) whereas clone TG9 contained two RGH, which shared significant similarity to RGH present in the sequenced tomato BAC clone AF411807L (Van der Hoeven et al. 2002) (Fig. 1). BAC clones Blb25 and TG77 were subsequently screened for the presence of RGH-specific sequences through polymerase chain reaction (PCR) analysis using the primers 4-PLOOP-F and 4-GLPL-R (Table 1), which were designed by aligning the RGH sequences of clone AF411807L with those present on BAC clone TG9. Southern analysis of BAC clones Blb22, Blb25, TG9, and TG77 using an RGH-specific PCR fragment amplified from BAC clone Blb25 as a probe identified a minimum of two RGH on BAC Blb25 and TG9 and one RGH on TG77 located on the overlapping part with TG9 (Fig. 1).

Libraries harboring random overlapping binary subclones of 8 to 10 kb were generated from BAC clones Blb25 and TG9. In total, 1,152 clones per library were screened for the presence of RGH using primers 4-GLO2-F and 4-GLO2-R (Table 1). Based on restriction analyses of the PCR fragments, RGH-positive subclones were divided into separate classes: *Blb3GH-A* and *Blb3GH-B* for *Rpi-blb3* and *AbptGH-A* and *AbptGH-B* for *Rpi-abpt*. After determining the relative positions of the RGH within the 8- to 13-kb subclones, candidates from each class were tar-

geted for transformation to the susceptible potato cv. Desiree. Transformation experiments carried out with subclones harboring candidates *Blb3GH-A*, *Blb3GH-B*, *AbptGH-A*, and *AbptGH-B* resulted in numerous primary transformants. Detached leaf assays using isolates IPO-0 and 90128 revealed that all of the plants transformed with subclones harboring candidates *Blb3GH-A*, *AbptGH-A*, and *AbptGH-B* were susceptible to both isolates but that the majority (seven of eight) of the tested transgenic plants harboring *Blb3GH-B* reacted to both isolates with an HR (Fig. 2). In view of the differential response between the primary transformants harboring *Blb3GH-A* and *Blb3GH-B*, we designated *Blb3GH-B* as the *Rpi-blb3* gene.

In order to identify additional candidate genes for *Rpi-abpt*, the *Rpi-abpt*-specific BAC library was screened with TG77-S, leading to the identification of the TG77 overlapping BAC clone TG92 (Fig. 1B). Screening of this BAC clone with different sets of primers designed to amplify *AbptGH-A*, *AbptGH-B*, *Blb3GH-A*, or *Rpi-blb3* resulted in the identification of a third *Rpi-abpt* candidate gene, *AbptGH-C* (Fig. 1B). The *AbptGH-C* LRR domain was amplified, cloned, and sequenced using the primers Blb3-LRR-1-8 (Table 1). Sequence comparison of the three *abpt*-GHs enabled us to design an *AbptGH-C*-specific marker (Fig. 1B) which cosegregated with resistance. Southern blot analysis using the *AbptGH-C* ampli-

Fig. 1. Genetic and physical map of the **A**, *Rpi-blb3* and **B**, *Rpi-abpt* loci. Indicated are the relative positions of markers (in green are the one used in the mapping of both genes), the number of recombinants identified between markers, the overlapping BAC clones defining the two loci, and the relative positions of the resistance (*R*) gene candidates that have been targeted for complementation analysis.

con as a probe revealed that clone TG92 contained one resistance gene encompassing an LRR domain highly similar to the one of *AbptGH-C*. Primers designed on the start and stop codon of *Rpi-blb3* were subsequently used to amplify a full-length *AbptGH-C* amplicon from clone TG92, which was cloned into the Gateway entry vector pDONR221. Using Multisite Gateway technology, the *AbptGH-C* amplicon was subsequently cloned into the binary pKGW-MG destination vector between *Rpi-blb3*-derived promoter and terminator sequences of 2,723 and 883 nucleotides, which were cloned into pDONR P4-P1R and pDONR P2R-P3 (Supplementary Fig. S1).

Complementation analysis was carried out in *N. benthamiana* using the *Agrobacterium tumefaciens* transient assay, whereby leaves from 4-week-old wild-type *N. benthamiana* plants were infiltrated with the *Agrobacterium* COR308 containing pKGW-*AbptGH-C*. The binary clones pBP-*Rpi-blb3* and pKGW-*Rpi-blb3*, comprising the genomic *Rpi-blb3* gene and a Multisite Gateway reconstituted *Rpi-blb3* gene construct, respectively, were used as positive controls and pBP-*AbptGH-A* as a negative control. After 2 days, infiltrated leaves were inoculated with *P. infestans* strains PY23, IPO-0, and IPO-C in detached-leaf assays. Leaves infiltrated with pKGW-*AbptGH-C*, pBP-*Rpi-blb3*, and pKGW-*Rpi-blb3* developed HR at the inoculation sites whereas noninfiltrated leaves and those infiltrated with pBP-*AbptGH-A* were susceptible to isolate PY23 and IPO-0. As expected, all leaves inoculated with IPO-C were susceptible (Fig. 3; Table 2). In view of these results, *AbptGH-C* was designated *Rpi-abpt*.

Allele mining for *R2* and *R2-like*.

Rpi-blb3 and *Rpi-abpt* belong to the major late blight resistance locus on LG IV that also harbors *R2* and *R2-like* (Li et

al. 1998; Park et al. 2005c). In view of the conserved marker order and observed allelic conservation between the genetic maps of *Rpi-blb3*, *Rpi-abpt*, *R2*, and *R2-like* (Park et al. 2005c) and the high sequence conservation between *Rpi-blb3* and *Rpi-abpt*, we hypothesized that *R2* and *R2-like* were orthologues of *Rpi-blb3* and, thus, attempted to clone them through an allelic mining strategy. The same primers used to amplify the *Rpi-abpt* candidate gene from BAC clone TG92 were used to amplify full-length *Blb3GH* from the resistant parental genotypes Cebeco44158-4 (MaR2) and AM3778-16 harboring *R2* and *R2-like*, respectively. Amplicons of the expected size were cloned into pDONR221 and fully sequenced. In total, 8 unique sequences were obtained from AM3778-16 (*R2-likeGH*) and 19 from Cebeco44158-4 (*R2GH*), with amino acid identities between *Rpi-blb3* and the novel *Blb3GH* ranging from 86.4 to 97.3% for *R2-likeGH* and 83.8 to 94.2% for *R2GH* (Supplementary Tables S1 and S2). Phylogenetic analysis of all the available amino acid sequences clustered one *R2-likeGH* and five *R2GH* in a clade together with the functional proteins *Rpi-blb3* and *Rpi-abpt* (Fig. 4). The amino acid sequence of *R2-likeGH-8* shares 97.3% amino acid identity with *Rpi-blb3*. *R2GH-2*, *R2GH-8*, *R2GH-G3*, *R2GH-D3*, and *R2GH-65* share 94.2, 91, 92.6, 89.7, and 92.8% amino acid identity, respectively, with *Rpi-blb3*. This set of candidate genes was targeted for functional analysis and, therefore, cloned into the binary vector pKGW-MG between the *Rpi-blb3* promoter and terminator sequences, as described above for the *Rpi-abpt* gene.

Transient complementation assays in *N. benthamiana* indicated that *R2GH-G3* and *R2-likeGH-8* conferred resistance to Pi isolate IPO-0, whereas *R2GH-2*, *R2GH-8*, *R2GH-D3*, and *R2GH-65* were nonfunctional (Fig. 3). Therefore, *R2GH-G3* and *R2-likeGH-8* were designated *R2* and *R2-like*, respectively.

Table 1. Overview of markers and primers used for mapping and cloning of *Rpi-blb3*

Name	Type	Polymerase chain reaction primer (5' to 3')	Tm ^a	Enzyme ^b
Th21	SCAR	F: ATTCAAAATTCTAGTTCCGCC R: AACGGCAAAAAAGCACCAC	...	a.s.
Blb22-S	CAPS	F: GTTTGATGTATGTTTGTCTTGC R: TAATGCACTAATACTAAGT	56	MspI
Blb22-T	SCAR	F: CTTTATTAGTTCCAAGAGCTAC R: ACCCATCCCTTTTCCTTATC	56	...
Blb25-S	CAPS	F: ACAGATGCTACGTCCATCAC R: CTCACATGCGATGCAAAAAG	56	AluI
Blb25-T	CAPS	F: TTTCGATTATGGTGAGCCTTC R: TAGAAAAAGGGTGGTTGTGAC	56	Hpy 188
RGH1	CAPS	F: GGSAAAGACCACTCTTGCAAG R: GGTTTTAAAGCTGCTAATGTTG	50	HpyCH4IV
RGH2	SCAR	F: GGSAAAGACCACTCTTGCAAG R: TGGTYATAATYACTCTGCTGC	50	a.s.
RGH3	CAPS	F: ATGRCTGATGCMTTTTRTGTC R: CCYAAAGTASAGAAAACACTGC	50	HaeIII
4-PLOOP	...	F: GGATGGGGIGGiYTiGGiARGAC	68	...
4-GLPL	...	R: TACiACAATiGCAAGiGGTAAMCC	...	...
4-GLO2	...	F: GTGTCTCTCAAGAGTACAACAC R: GCTCGAACATCAAGTAGTTCC	56	...
Blb3-start	...	F: ATGGCTGATGCCTTTCTRTCATTTG R: TCAGGAATCTCCTTTAAATTTGGAC	55	...
Blb3-end	...	F: TCTTCCTTAGCATTCGTAGC R: CTTTAGGAATACTAGTTTGTATTG	55	...
Blb3-prom	...	F: AGCTTTTCTGCCAAGCACATTGG R: GTACCTCCGTTTGTGTTTGATC	55	...
Blb3-ter	...	F: CTCTTTATGTATCAGACATGGC R: CAACATCTTCCACTGATCAC	55	...
Blb3-LRR-1-8	...	F: CCCCAGTTGTATAATGGTTG R: TGCTTGAGTGATTGAATCTCC	55	...
Blb3-prom -end	...	R: GGCCATATTCAGACTGGGAG F: AGCTTTTGTAGTGTGTAATTGG	55	...
Blb3-orf-bg	...	R: GTAACACGGACTCGAGGG	...	...
Sto-orf-bg	...			
Blb3-spe	...			

^a Annealing temperature. In AF411807R, annealing temperature was 48°C for the first seven cycles and 54°C for the last 30 cycles.

^b Restriction enzymes that reveal polymorphism between resistant and susceptible linked alleles of the marker; a.s.: allele-specific marker showing polymorphism without digestion.

Fig. 2. Genetic complementation for late blight resistance. Eight days after inoculation with *Phytophthora infestans* isolate 90128, large necrotic lesions and massive sporulation were noted for potato cv. Desiree, and derived transformant Blb25A-2-4 and Blb25A-2-5, both expressing resistance gene homologue RGH-Blb25A. Typical resistance phenotype (i.e., hypersensitive response) was observed for Blb25B-2-1 and Blb25B-2-2 expressing RGH-Blb25B (*Rpi-blb3*).

All four *R* genes interact with the RXLR-EER effector *PiAVR2*.

Using a map-based cloning approach, markers flanking the *PiAVR2* locus (van der Lee et al. 1997) were used to delimit a physical region of 742,635 bp of the *P. infestans* genome sequence spanning the genetic interval containing *PiAVR2*. Within this region, two candidate *PiAVR2* genes on supercontig 16 of the *P. infestans* genome (16_7987 and 16_8044), each encoding a secreted RXLR-EER effector, were identified. The cloning and analyses of these gene candidates will be reported in detail in a separate publication (E. Gilroy, *personal communication*).

To functionally analyze the two RxLR-EER candidate genes identified at the *PiAVR2* locus in this study, *N. benthamiana* leaves were co-infiltrated with *A. tumefaciens* strains carrying a binary construct expressing one of the four *Rpi* genes under study together with strains carrying a binary construct expressing each of the two candidate RxLR-EER genes. Co-infiltration of *Rpi-blb3*, *Rpi-abpt*, *R2*, and *R2-like* with 16_7987 resulted in a hypersensitive cell death response (Fig. 5), whereas co-expression of these *Rpi* genes with the 16_8044 candidate or *PiAVR3a* failed to elicit visible cell death (Fig. 5) (E. Gilroy, *personal communication*). Based on these results, 16_7987 was designated *PiAVR2* and, clearly, all four *Rpi* genes under study interact with *PiAVR2*.

Gene structure and functionality.

Rpi-blb3, *Rpi-abpt*, *R2*, and *R2-like* are intronless genes which encode open reading frames (ORF) of 2,538 to 2,544 nucleotides that code for proteins of 845 to 847 amino acids harboring all the signature sequences characteristic of leucine

Fig. 3. Complementation of *Rpi-blb3*, *Rpi-abpt*, *R2*, *R2-like*, and *Rpi-sto1* by transient expression in *Nicotiana benthamiana*, followed by inoculation by *Phytophthora infestans* isolate Ipo0 (avirulent) and Ipo-C (virulent on all except *Rpi-sto1*). The prefix pBP stand for pBINplus, and pKGW stand for the gateway destination vector containing *Rpi-blb3* regulatory elements. In leaves expressing pB-Rpi-blb3, pKGW-Rpi-blb3, pKGW-Rpi-abpt, pKGW-R2-like, and pKGW-R2, a hypersensitive response was observed at 7 days postinoculation with *P. infestans* isolate Ipo-0, whereas large necrotic lesions and massive sporulation were evident for *P. infestans* isolate Ipo-C. Wild-type *N. benthamiana* (Control*) and the nonfunctional AbptGH-A (pBP-AbptGH-A) were included as susceptible controls, and *Rpi-sto1* (pKGW-Rpi-sto1) was included as a resistant control.

Table 2. Overview of resistance screening with a set of 16 *Phytophthora infestans* isolates^a

Species	Genotype	Gene	Chr.	py 23	IPO-0	90128	H30P04	VK98014	IPO428-2	Dintel	Katshaar	F95573	EC1	89148-09	99183	99189 ^b	IPO-C	USA618	99177
BLB	Blb99-256-3	<i>Rpi-blb3</i>	4	R	R	R	R	R	R	R	R	R	R	R	R	S	S	S	S
BLB	707TG11-1	<i>Rpi-abpt</i>	4	R	R	R	R	R	R	R	R	R	R	R	R	R	S	S	S
BLB	AM3778-16	<i>R2-like</i>	4	R	R	R	R	R	R	R	R	R	R	R	R	S	S	S	S
DMS	Cebeco44158-4	<i>R2</i>	4	R	R	R	R	Rq	R	Rq	R	R	R	R	R	S	S	S	S
cv. Desiree	...	...	...	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
cv. Bintje	...	...	...	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S

^a Differential specificity is in bold. Chr. = chromosome, S = susceptible, R = resistant, and Rq = quantitatively resistant.

^b Isolate differentially recognized.

Fig. 4. Phylogenetic tree at the amino acid level of Rpi-blb3; resistance (*R*) gene homologues (RGH) amplified from late-blight-resistant potato clones harboring *Rpi-abpt*, *R2-like*, or *R2*; RGH present on the tomato Bac clone AF411807, *RPP13-Nd*, and *Rpi-blb1*. The group containing Rpi-blb3, Rpi-abpt, R2, and R2-like is boxed.

Fig. 5. *Agrobacterium*-mediated coexpression of *PiAvr2* or *PiAvr3a* together with *Rpi-blb3*, *Rpi-abpt*, *R2*, *R2-like*, *Rpi-sto1*, the nonfunctional *abptGH-A*, or *R3a*. Different infiltration points are indicated on the map. On each leaf, four infiltration spots were performed, duplicated co-infiltration of the avirulence (*Avr*) and resistance (*R*) gene on the left, *Avr* on the top right and *R* gene on the bottom right. *Rpi-blb3*, *Rpi-abpt*, *R2*, and *R2-like* in combination with *PiAvr2* as well as *R3a* and *PiAvr3a* induced the hypersensitive response. Pictures were taken 6 days postinfiltration.

zipper (LZ)-NBS-LRR R proteins (Fig. 6). Interestingly, with respect to known functional R proteins, *Rpi-blb3*, *Rpi-abpt*, *R2*, and *R2-like* share the highest similarity (34.9% amino acids) with *RPP13* from *Arabidopsis thaliana* (Bittner-Eddy et al. 2000), which confers resistance to *H. arabidopsidis* isolates containing *ATR13*. The highest similarity with *RPP13* resides in the NBS domain, with 49.3% sequence identity, and the lowest within the LRR domain (34.3%). The LRR domains of *Rpi-blb3*, *Rpi-abpt*, *R2*, and *R2-like* are highly homologous and comprise 14 imperfect repeats (Fig. 6). The LZ and NBS domains are more polymorphic, those of *R2* being the most divergent. *Rpi-abpt* and *R2-like* are identical except for the sequence between LRR2 and LRR3, where *Rpi-abpt* contains a stretch of amino acids that is identical to that of *R2*. The LRR domain of *R2* is identical to that of *Rpi-abpt* (Fig. 6).

Alignment of the nucleotide sequences of the four functional genes and those of four additional *Blb3GHs* and subsequent analysis of informative polymorphic sites (IPS), (i.e., sites where two or more genes carry the same nucleotide) (Parniske et al. 1997) reveals clear blocks of sequence affiliation between different members of the gene family (Supplementary Fig. S2), indicating that sequence exchange events between *Blb3GHs* have been involved in the evolution of the gene family. Interestingly, the observed sequence affiliations in the 5' terminal half of the genes extend throughout the LZ and NBS domains whereas sequence affiliations in the LRR domain suggest exchange of specific combinations of LRR, underlining the modular nature of R proteins.

In an attempt to assess the biological relevance of the observed sequence exchange events in relation to resistance spectrum, detached leaves of the parental clones harboring *Rpi-blb3* (Blb99-256-3), *Rpi-abpt* (707TG11), *R2* (Cebeco44158-4 (MaR2)), and *R2-like* (AM3778-16) were inoculated with 16 different *P. infestans* isolates (Table 2). For 15 isolates, all four clones displayed the same specificity. However, one isolate, PIC99189, displayed a differential interaction; Blb99-256-3, AM3778-16, and Cebeco44158-4 (MaR2) were susceptible to this isolate whereas 707TG11-1 was resistant (Table 2). Previously, it was reported that 707TG11-1 contained tuber-specific *R1-like* (Park et al. 2005d) and foliage-specific *R3-haplotype* (Huang 2005). To dissect the resistance specificity of 707TG11-1, we inoculated 48 F1 progeny plants of the *Rpi-abpt* mapping population RH4x103 with isolates 90128 and PIC99189. For both isolates, a clear 1:1 segregation of resistance was observed but there was no correlation between resistance to both isolates, confirming that the resistance to isolate PIC99189 is, indeed, the result of a second gene other than *Rpi-abpt*, and not located on LG IV.

In order to confirm the functionality of *Rpi-abpt*, *R2-like*, and *R2*, transgenic *Desiree* were generated. Detached leaves of six, five, and two independent transgenic lines expressing *R2-like*, *Rpi-abpt*, and *R2*, respectively, were inoculated with *P. infestans* isolates IPO-0 and IPO-C. All transformants reacted with an HR to isolate IPO-0 and were susceptible to isolate IPO-C (Supplementary Fig. S3). This result confirms that *Rpi-abpt*, *R2-like*, and *R2* are functional R genes with expected specificity to *P. infestans* isolates. Moreover, the use of the *N. benthamiana* transient complementation assay as an efficient tool for rapid functional screen of putative R genes is verified.

DISCUSSION

In this study, we describe the cloning and functional characterization of four homologous R genes from the major late blight resistance locus on LG IV of potato. Previously, marker order and allelic conservation suggested that *Rpi-blb3*, *Rpi-abpt*, *R2*, and *R2-like* reside at the same locus on LG IV (Park

et al. 2005a, b, and c). *Rpi-blb3* and *Rpi-abpt* were cloned using a map-based cloning approach whereby physical maps of both loci were generated by screening appropriate BAC libraries with a highly linked molecular marker, followed by subsequent rescreening of the libraries with novel BAC end markers until the outer ends of the BAC contigs exceeded the genetic boundaries of the R loci. In the case of *Rpi-blb3*, two candidate NBS-LRR genes were identified and subcloned into a binary vector and targeted for complementation analysis through stable transformation of a susceptible potato cultivar.

For *Rpi-abpt*, an NBS-LRR candidate was cloned from the BAC using a homology-based PCR cloning strategy using primers designed on the functional *Rpi-blb3* gene. In addition, complementation was carried out using an *Agrobacterium*-mediated transient expression assay in *N. benthamiana* followed by detached leaf assays with appropriate *P. infestans* isolates (H. Rietman, unpublished data).

Development of the transient complementation assay together with the successful in planta expression of *Rpi-abpt* and other R genes (H. Rietman, unpublished data) using the DNA regulatory elements of *Rpi-blb3* facilitated the rapid cloning of *R2* and *R2-like* through an efficient functional allele-mining strategy. A prerequisite for such an allele-mining strategy is prior knowledge of candidate gene sequences at the locus of interest. Observations within the family *Solanaceae* indicate that R gene clusters are conserved across species, thus enabling comparative genomics (Grube et al. 2000; Huang. 2005; van der Vossen et al. 2005). As more and more R genes are mapped and cloned, the greater is the chance that novel R genes reside at known and well-characterized loci. Moreover, ongoing potato and tomato genome-sequencing projects by international consortia promise to provide a complete survey of the distribution of R gene clusters in the family *Solanaceae*, enabling even faster cloning of R genes through allele-mining strategies.

Interestingly, with respect to known functional R proteins, *Rpi-blb3*, *Rpi-abpt*, *R2*, and *R2-like* share the highest similarity (34.9% amino acids) with *RPP13* from *A. thaliana* (Bittner-Eddy et al. 2000), which confers resistance to *H. arabidopsidis*. Mining of *RPP13* alleles in a diverse set of *A. thaliana* accessions revealed both extreme sequence diversity and functional diversity in pathogen recognition (Rose et al. 2004). In line with this observation, a significant excess of amino acid polymorphism was localized within the LRR domain of *RPP13*. In the case of *Rpi-blb3*, *Rpi-abpt*, *R2*, and *R2-like*, the LRR domains are nearly identical despite significant polymorphism at the coiled-coil and NBS domains, suggesting conservation of function; and, indeed, we have shown that all four proteins interact with the same effector protein, PiAVR2, which was recently cloned through a map-based cloning approach (E. Gilroy, personal communication). Although the origin of *Rpi-abpt* and *R2-like* remain unclear, this finding is a second example of how R gene recognition specificities can be conserved across *Solanum* spp. originated from different potato superseries; in this case, *S. bulbocastanum* (*Rpi-blb3*) belongs to the *Potato Stellata Bulbocastana* and *S. demissum* (*R2*) to the *Potato Rotata demissa*. Recently, we demonstrated both gene conservation and specificity conservation between the *S. bulbocastanum*-derived gene *Rpi-blb1* and the *S. stoloniferum*- and *S. papita*-derived orthologues *Rpi-sto1* and *Rpi-pa1*, respectively (Vleeshouwers et al. 2008). Despite apparent positive selection in the LRR domain, the LZ and NBS domains are more polymorphic, those of *R2* being the most divergent. Clear blocks of sequence affiliation between the four functional R proteins and additional *Rpi-blb3* gene homologs suggest exchange of LZ, NBS, and LRR domains and also of specific LRR, underlining the modular nature of these proteins. *R2* has a particular gene structure likely to be the

Rpi-blb3	MADAFLSFAVQKLGDFLIQKVSLRKS	LRDEIRWLINELLFIRSF	LRDAEQKQCGDQRVQQ	60				
R2-like				60				
Rpi-abpt				60				
R2		Q N K E R Q L Y		60				
Rpi-blb3	WVFEIN	SIANDAVAI	LETYSFEAGKGASRLKACTCICRKEKKFYNVAAEIQSLKQRIMDI	120				
R2-like				120				
Rpi-abpt				120				
R2		V T NBS A YT		120				
Rpi-blb3	SRKRETYGITNINYNSGERPSNQVTT	TLRRTTSYVDEQDYIFVGFQDVVQTLLAQLLKAEP		180				
R2-like		N A G K F		180				
Rpi-abpt		N A G K F		180				
R2		N S G R D L K		180				
Rpi-blb3	RRSVLSIYGMGGLGKTTL	ARKLYTSPDILNSFPTRAWICVSQEYNTMDLLRTIIKSIQGC		240				
R2-like			R N R	240				
Rpi-abpt			R N R	240				
R2	T H	N SA	N V R	240				
Rpi-blb3	AKETLDLLEKMAEIDLENHLRD	LLKECKYL	VVDDVWQREAWESLKRAFPDGKNGSRVII	300				
R2-like	T	R T G IY R	S	300				
Rpi-abpt	T	R T G IY R	S	300				
R2	T	R T G IY R M K D S		300				
Rpi-blb3	TTRKEDVAERVDHRGFVHKLRFLS	QEE	SWDLFRRKLLDVRAMVP	EMESLAKDMVEKCRGL	360			
R2-like		A D			360			
Rpi-abpt		A D			360			
R2	Q A DI		K S N		360			
Rpi-blb3	PLAIVVLSGLLSHKKGLNQWQKV	KDHLWKNIKEDKSIEISNILS	SYNDLSTALKQCFLY		420			
R2-like					420			
Rpi-abpt					420			
R2					420			
Rpi-blb3	FGIFPEDQVVKADDIIRLWMAEGFIPRGEER	MEDVADGFLNELIR	SLVQVAKTFWEKVT		480			
R2-like					480			
Rpi-abpt					480			
R2					480			
Rpi-blb3	DCRVHDLRLDLAIQKALEVNF	FDVYGRSHS	IS	SSLCIRHGIHSEGERYLS	SLDL	SNL	KLR	540
R2-like			I D					540
Rpi-abpt			I D					540
R2		V	I D					540
Rpi-blb3	SIMFFDPDFR-KMSHINLRSEFQHL	YVLYLDTNFGYVSMVPDAIGSLYHLKLLRLRGIHD						599
R2-like								599
Rpi-abpt		YICNVFQ DV---	R					597
R2		YICNVFQ DV---	R					597
Rpi-blb3	IPSSIGNLKNLQTLVVVNGY	TFFCQLPCKTADLINLRHLVVOYSEPLKCINKL	TSLOVLD					659
R2-like			E T					659
Rpi-abpt			E T					657
R2			E T					657
Rpi-blb3	GVACDQWKDVDPVDLVNLR	ELSM	DRIRSSYSLN	NISS	LN	SLK	NLSTLKLICGERQSFASLEFV	719
R2-like								719
Rpi-abpt								717
R2								717
Rpi-blb3	NCCEKLQKLWLQGR	IEELPHLFNSITMVL	SFSELTEDPMPILGRFPNLRN	NL	KLDGAYE			779
R2-like								779
Rpi-abpt								777
R2								777
Rpi-blb3	GKEIMCSDNSFSQLEFLH	LRDLWKLERWDLGTSAM	PLIKGLGIHNC	PNLKEIPERM	KDME			839
R2-like						V		839
Rpi-abpt						V		837
R2						V		837
Rpi-blb3	LLKRN	YML						847
R2-like								847
Rpi-abpt								845
R2								845

Fig. 6. Alignment of the deduced protein product encoded by the functional genes *Rpi-blb3*, *Rpi-abpt*, *R2-like*, and *R2*. The full amino acid sequence of *Rpi-blb3* and in comparison, differing amino acids of *Rpi-abpt*, *R2-like*, and *R2* are shown. In italics is the leucine zipper domain with the first and the fifth hydrophobic residues from the heptads in bold and underlined, respectively. Positions of the nucleotide binding (NB)-ARC and leucine-rich repeat are indicated by arrows. The NB-ARC domain has its characteristic conserved domains highlighted in gray (kinase 1a, kinase 2, and kinase 3). The leucine-rich repeat domain is composed of 14 repeats with the motif xxLxLxxx.

product of a relatively recent recombination event. The LZ-NBS domain (nucleotides 1 to 1,149) is highly affiliated to that of the nonfunctional *R2-GH-D3*, whereas the LRR domain (nucleotides 1,059 to 2,544) is highly affiliated to that of *Rpi-abpt* and the nonfunctional *R2-GH-65*, revealing a sequence exchange track between nucleotides 1,059 and 1,149. Sequence exchange between RGH has been proposed as a major mechanism shaping *R* gene diversity in plants (Kuang et al. 2004; Meyers et al. 1999; Michelmore and Meyers 1998). Interestingly, the approximate position of this recombination site corresponds to the one described by Rose and associates (2004) for the *RPP13* allele *ksk-2*, a region between the conserved GLPL and VHD motifs of the NB-ARC domain. This is in line with the expectation that, in multidomain proteins, sequence exchange tracks will be concentrated at specific sites that separate domains with independent functions.

Preliminary results from ongoing *Rpi-blb3* allele-mining studies in *Solanum* germplasm indicate that *Rpi-blb3* is present in several other species (e.g., *S. brachistotrichum* and *S. pinnatisectum*). In addition several *Rpi* genes recently identified in our laboratory (data not shown) also map to the *Rpi-blb3* interval. The quantitative trait locus (QTL) for late blight resistance (Pi-QTL) (Sandbrink et al. 2000), explaining 40% of the phenotypic variation, was mapped to the *Rpi-blb3* locus and renamed *Rpi-mcd1* (Tan et al. 2008). Similarly, high-resolution mapping data of the major Pi-QTL identified in cv. Stirling strongly suggest that this Pi-QTL is also an *Rpi-blb3* homologue (Hein et al. 2007) (I. Hein, *personal communication*). In addition, various other examples of Pi-QTL on the short arm of LG IV have been described (Bradshaw et al. 2004, 2006; Leonards-Schippers et al. 1994; Sandbrink et al. 2000).

Rpi-blb3, *Rpi-abpt*, *R2*, and *R2*-like specifically detect PiAVR2. Do the *Rpi-blb3* homologues putatively underlying the Pi-QTL described above also recognize PiAVR2 homologues? If so, do they weakly recognize an allele that is conserved throughout *P. infestans* or do they recognize multiple alleles, thus resulting in nonrace-specific resistance?

Current models for NB-LRR R proteins suggest a dual role for the LRR domain, not only as recognition specificity determinant but also as a repressor of inappropriate NB activation (Belkadir et al. 2004). Furthermore, evolutionary analyses of R proteins and their structure has shown selection pressure on the LRR region but also on other domains within R proteins which, in light of the dual function, may point toward coevolution in R proteins to establish and maintain recognition of effectors while maintaining the inhibitory function (McDowell and Simon 2006). Our findings are slightly different, contradictory, indicating conservation in the LRR domain but more variation in the NBS domain, that may point toward different signaling pathways or additional effectors being recognized by these R proteins.

MATERIALS AND METHODS

Plant material and *P. infestans* isolates.

In this study, we used the four resistant parental clones Blb99-256-3 harboring *Rpi-blb3* (Park et al. 2005a), 707TG11-1 *Rpi-abpt* (Park et al. 2005c), Cebeco44158-4 (MaR2), *R2*, and AM3778-16 *R2-like* (Park et al. 2005b). F1 progeny plants of population RH4x103 were derived from a cross between the late-blight-resistant clone 707TG11-1 and the diploid potato clone RH89-039-16 (Park et al. 2005c). The susceptible potato cv. Desiree was used for *Agrobacterium*-mediated transformation. The characteristics and origin of *P. infestans* isolates used in this study are indicated in Supplementary Table S3.

BAC library construction.

Clones Blb99-256-3 and 707TG11-1 were used as DNA sources for the construction of BAC libraries. High molecular weight DNA preparation and BAC library construction were carried out as described previously (Roupe van der Voort et al. 1999). Approximately 74,000 clones with an average insert size of 85 kb, corresponding to eight genome equivalents, were obtained per library. The BAC clones were stored as bacterial pools containing approximately 700 to 1,000 white colonies. These were generated by scraping the colonies from the agar plates into Luria-Bertani (LB) medium containing 18% glycerol and chloramphenicol at 12.5 $\mu\text{g ml}^{-1}$ using a sterile glass spreader. These so-called super pools were stored at -80°C . Marker screening of the BAC libraries was done, first by isolating plasmid DNA from each pool using the standard alkaline lysis protocol, and PCR was carried out to identify positive pools. Bacteria corresponding to positive pools were diluted and plated on LB agar plate containing chloramphenicol (12.5 $\mu\text{g ml}^{-1}$). Individual white colonies were picked into 384-well microtiter plates and single positive BAC clones were subsequently identified by marker screening as described by Roupe van der Voort and associates (1999). Names of BAC clones isolated from the super pools carry the prefix Blb (e.g., Blb25) or TG (e.g., TG9).

Subcloning of candidate genes.

Candidate RGH were subcloned from BAC clone BLB25 and TG9 as follows. Aliquots of approximately 1 μg of BAC DNA were digested with 0.03 U of *Sau3A*I restriction enzyme for 10 min. The partially digested BAC DNA was subjected to electrophoresis at room temperature in 0.5 $\times$ Tris-acetate EDTA using a linear increasing pulse time of 1 to 10 s and a field strength of 90 V cm^{-1} for 6 h. After electrophoresis, the agarose gel was stained with ethidium bromide to locate the region of the gel containing DNA fragments of approximately 10 kbp in size. This region was excised from the gel and treated with GELASE (Epicentre Technologies, Madison, WI, U.S.A.) according to the manufacturer's instructions. The size-selected DNA was ligated to the *Bam*HI-digested and dephosphorylated binary vector pBINPLUS (van Engelen et al. 1995) followed by transformation to Electro-MAX *Escherichia coli* DH10B competent cells (Life Technologies, Paisley, U.K.).

DNA sequencing and computer analysis.

BAC clone sequencing was carried out using a shotgun cloning strategy. Sequencing reactions were performed using a dye terminator cycle sequencing reaction kit (Perkin-Elmer, Pt Biosystem, Warrington, U.K.), M13 universal forward and reverse primers, and an ABI377 automated sequencer (Applied Biosystem, La Jolla, CA, U.S.A.). Sequence contigs were assembled using the STADEN sequence and analysis program (Dear and Staden 1991).

The binary constructs were sequenced using a primer walking strategy (700 by 700 bp). Gene structures were predicted using FGENESH++ (Softberry) and protein sequences were deduced by translation of the ORF using the standard genetic code. Multiple sequence alignments were conducted using CLUSTALX 1.81 (Thompson et al. 1997). The search for genes homologous to *Rpi-blb3* was carried out using the Basic Local Alignment Search Tool (BLAST). Conserved domains were identified using Swiss-Prot (InterProScan, EMBL-EBI, ExPASy, SAPS).

Resistance assay.

Detached leaf assays were used to determine the resistance phenotypes of primary transformants of potato and transiently

transformed *N. benthamiana* leaves. For complementation analyses, primary transformants were tested with isolates IPO-0 and 90128 (Fig. 2). Inoculum preparations and inoculations were performed as described by Vleeshouwers and associates (1999). Six days after inoculation, plant phenotypes were determined. Leaves showing no symptoms or a localized necrosis at the point of inoculation were scored as resistant and those with clear sporulating lesions (visible by naked eye) as susceptible.

Transient complementation in *N. benthamiana*.

Infiltrations of *Agrobacterium* strains carrying the candidate RGH were performed in *N. benthamiana*. Recombinant COR308 *Agrobacterium tumefaciens* cultures were grown in LB medium (10 g of bacteriological peptone, 10 g of NaCl, and 5 g of yeast extract in 1 liter of milli-Q water) supplemented with tetracycline at 5 mg/liter and kanamycin at 50 mg/liter. After 1 or 2 days, a calculated amount of culture (according to an optical density at 600 nm [OD₆₀₀] of 0.5) was transferred to YEB medium (5 g of beef extract, 5 g of bacteriological peptone, 5 g of sucrose, 1 g of yeast extract, and 2 ml of 1 M MgSO₄ in 1 liter of milli-Q water) supplemented with kanamycin for all strains. After 1 day, overnight cells were centrifuged at 3,500 rpm and resuspended in MMA medium (20 g of sucrose, 5 g of MS salts, and 1.95 g of morpholineethanesulfonic acid) supplemented with 1 ml of 200 mM acetosyringone to a final OD₆₀₀ of 0.2 and infiltrated into leaves of 4-week-old plants with a 3-ml syringe. After 2 days, infiltrated leaves were detached and inoculated with *P. infestans* strains IPO-C and PY23 in detached-leaf assays. HR or *P. infestans* sporulation was observed between 4 and 8 days postinoculation.

Agrobacterium-mediated coexpression in *N. benthamiana*.

pBP-Rpiblb3, *pKGW-Rpi-blb3*, *pKGW-Rpi-abpt*, *pKGW-R2*, *pKGW-R2-like*, *pKGW-Rpi-sto1*, *pBP-abptGH-A*, and *pBP-R3a* were transferred to *A. tumefaciens* AGL1 (Lazo et al. 1991) containing the helper plasmid pVirG and pSoup (van der Fits et al. 2000). Recombinant *A. tumefaciens* cultures were grown in LB medium supplemented with tetracycline at 5 mg/liter, carbenicillin at 100 mg/liter, chloramphenicol at 75 mg/liter, and kanamycin at 50 mg/liter. The avirulence effectors were cloned into the pGRAB vector (kindly supplied by Sean Chapman, SCRI, Scotland, U.K.) and transferred to the same *A. tumefaciens* AGL1. Recombinant *A. tumefaciens* cultures were grown in LB medium supplemented with chloramphenicol at 30 mg/liter and kanamycin at 50 mg/liter. The same protocol as for the transient complementation was used for the subcultures. *PiAVR Agrobacterium* culture and *Rpi-gene Agrobacterium* culture were mixed at a final OD₆₀₀ of 0.1 for each and infiltrated into leaves from 5- to 7-week-old plants. Specific recognition of the AVR gene by the R gene induces HR from 5 days postinfiltration.

Transformation of susceptible potato cultivar.

Binary plasmids harboring the candidate genes *Blb3GH-A*, *Blb3GH-B*, *AbptGH-A*, and *AbptGH-B* were transferred to *A. tumefaciens* COR308 (Hamilton et al. 1996) and the constructs *pKGW-Rpi-abpt*, *pKGW-R2-like*, and *pKGW-R2* in AGL1 (Lazo et al. 1991). After verifying their stability in *Agrobacterium* spp., these clones were transformed to the susceptible potato cv. Desiree. Overnight cultures of the transformed *A. tumefaciens* strain were used to transform internodal cuttings from in vitro grown plants (Heilersig et al. 2006). A total of 100 to 200 explants were used for each transformation. Three to eight plants per transformant were transferred to the greenhouse.

Allele mining.

Primers of 32 nucleotides were designed on the *Rpi-blb3* sequence, with the forward primer beginning at the start codon (*Blb3*-start) and the reverse primer beginning at the stop codon (*Blb3*-end) (Table 1). The BAC clone TG92 containing *Rpi-abpt* and the genomic DNA of the parental resistant genotype AM-(3778-16) containing *R2-like* were used as template in a long-range PCR reaction (95°C for 2 min 40 s; 30× [94°C for 20 min, 56.8°C for 25 min, and 64.3°C for 7min], and 64.3°C for 25 min) using the high-fidelity DNA polymerase *PfuTurbo* (Stratagene, La Jolla, CA, U.S.A.). PCR products were separated on agarose gel and purified using the QIAquick gel extraction kit from Qiagen (Hilden, Germany). The purified pool of *R2-likeGH* ORF and the purified *Rpi-abpt* ORF were used in a BP reaction together with the donor plasmid pDONR 221 according to the protocol described by Untergasser (2006b). Subsequently, the BP reaction products were transferred to DH10B *E. coli* competent cells and plated on LB agar plates containing the antibiotic kanamycin at the concentration of 100 µg/ml. Colonies were cultured in LB liquid medium supplemented with kanamycin. Plasmid DNA was subsequently extracted by mini-prep protocol adapted from Sambrook and associates (1989) using the P1, P2, and P3 solutions from Qiagen. The first and last 700 bp of plasmids containing *R2-likeGH* ORF were sequenced and classified. Then, one clone of each group was entirely sequenced and compared with the *Rpi-blb3* sequence. The clones sharing high similarity with *Rpi-blb3* were cloned into the destination vector pKGW-MG (kindly provided by Andreas Untergasser) in between *Rpi-blb3* regulatory elements via a multiple LR reaction with the *Rpi-blb3* promoter in pDONR-P4P1R, the *Rpi-blb3* terminator in pDONR-P2RP3, the ORF of interest in pDONR221, and the binary destination using the protocol of Untergasser (2006a). The final expression constructs were then transferred to *A. tumefaciens* COR308 after the configuration of the constructs was confirmed by PCR.

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