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Phytophthora infestans Isolates Lacking Class I *ipiO* Variants Are Virulent on *Rpi-blb1* Potato

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A strategy to control the devastating late blight disease is providing potato cultivars with genes that are effective in resistance to a broad spectrum of *Phytophthora infestans* isolates. Thus far, most late blight resistance (*R*) genes that were introgressed in potato were quickly defeated. In contrast, the *Rpi-blb1* gene originating from *Solanum bulbocastanum* has performed as an exclusive broad-spectrum *R* gene for many years. Recently, the RXLR effector family *ipiO* was identified to contain *Avr-blb1*. Monitoring the genetic diversity of the *ipiO* family in a large set of isolates of *P. infestans* and related species resulted in 16 *ipiO* variants in three distinct classes. Class I and class II but not class III *ipiO* variants induce cell death when coinfiltrated with *Rpi-blb1* in *Nicotiana benthamiana*. Class I is highly diverse and is represented in all analyzed *P. infestans* isolates except two Mexican *P. infestans* isolates, and these were found virulent on *Rpi-blb1* plants. In its C-terminal domain, IPI-O contains a W motif that is essential for triggering Rpi-blb1–mediated cell death and is under positive selection. This study shows that profiling the variation of *Avr-blb1* within a *P. infestans* population is instrumental for predicting the effectiveness of *Rpi-blb1*–mediated resistance in potato.

Late blight caused by the oomycete *Phytophthora infestans* is one of the most severe threats to potato production worldwide (Fry 2008). Despite many efforts, effective methods to control late blight epidemics are still not available. In recent years, however, intensified research on both the pathogen and the host plant has deepened our insight into the molecular basis of virulence and avirulence determinants in *P. infestans* and of host defense responses. This knowledge is instrumental in obtaining genetic resistance in potato (Bryan and Hein 2008; Govers and Gijzen 2006; Park et al. 2009).

In order to invade plant cells without hindrance, pathogens secrete effector proteins that can manipulate host defense responses, thus resulting in effector-triggered susceptibility (ETS) (Jones and Dangl 2006; Kamoun 2006, 2007). When an effector is recognized by a resistance (R) protein, effector triggered immunity (ETI) is activated, often resulting in a hypersensitive response (HR). Effectors then act as avirulence (Avr) factors and the encoding *Avr* genes interact with *R* genes according to the gene-for-gene model (Flor 1971). The molecular arms race between the pathogen and its host drives coevolution of *R-Avr* gene pairs, as has been clearly demonstrated with the *Arabidopsis R* gene *RPP13* and the corresponding *Avr* factor *ATR13* of the oomycete *Hyaloperonospora arabidopsidis* (formerly *H. parasitica*) (Allen et al. 2004). Both *RPP13* and *ATR13* are highly variable, and by examining natural variants of ATR13, key amino acids were identified that are functionally essential for interaction with RPP13 (Allen et al. 2008). In *P. infestans*, only a few *Avr* genes have so far been studied at the molecular level. *Avr3a*, the counterpart of the *Solanum demissum* gene *R3a*, has two alleles—a virulent one and an avirulent one—that differ only two amino acids (Armstrong et al. 2005). The latter, $Avr3a^{KI}$, not only triggers a *R3a*-dependent HR, it is also able to suppress a cell-death response induced by the elicitin INF1. These two activities, however, are conditioned by distinct amino acids (Bos et al. 2009). On the plant side, *R3a* unleashed its evolutionary potential with numerous *R3a*-like genes, which resulted in a major late blight locus on chromosome 11 of *Solanum demissum* (Friedman and Baker 2007; Huang 2005; Huang et al. 2005). *Avr4*, which interacts with *S. demissum R4*, has one predominant avirulent allele in nature but, unlike *Avr3a*, the virulent allele has frameshift mutations and can no longer produce an effector protein (van Poppel et al. 2008). Recently, we described the identification of another potential *P. infestans Avr* gene, i.e., *ipiO*, the in planta–induced gene that was postulated as being involved in pathogenicity based on its expression profile (van West et al. 1998). The identification of *ipiO* as *Avr-blb1* resulted from an effector genomics approach that is based on high throughput functional profiling of effector genes in plants carrying *R* genes (Vleeshouwers et al. 2008). In the effector screening, two variants of *ipiO*, i.e., *ipiO1* and *ipiO2*, triggered a cell-death response in *S. bulbocastanum* plants carrying the late blight *R* gene *Rpi-blb1* (alternatively named RB) (Song et

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al. 2003; van der Vossen et al. 2003; Vleeshouwers et al. 2008). Cell-death responses to *ipiO1* and *ipiO2* were also noted in *S. stoloniferum*, which is the source of the *Rpi-blb1* homologs *Rpi-sto1* and *Rpi-pta1*. Accordingly, agro-coinfiltration of *Rpi-blb1*, *Rpi-sto1*, and *Rpi-pta1* with *ipiO1* and *ipiO2* in *Nicotiana benthamiana* resulted in specific cell death and provided functional evidence for the *R-Avr* interaction. However, *ipiO4*—a genetically more distant variant—did not elicit cell death when agro-coinfiltrated with *Rpi-blb1* nor with its homologs. This suggested that alleles or variants of the *ipiO* gene family vary with respect to recognition by *Rpi-blb1* and hence in avirulence to *Rpi-blb1*.

Like Avr3a and Avr4, IPI-O contains at its N-terminus a signal peptide for type II secretion and a RXLR-dEER motif for host-cell internalization, whereas the C-terminal domain is required for effector functions (Govers and Bouwmeester 2008; Rehmany et al. 2005; Whisson et al. 2007). The RXLR domain of IPI-O partly overlaps with a RGD cell adhesion motif, which has been shown to bind to a lectin receptor kinase in *Arabidopsis* that may function as an effector target (Gouget et al. 2006). This lectin receptor kinase participates in protein-protein interactions to mediate cell wall–plasma membrane adhesions, and it has been observed that IPI-O can disrupt these adhesions (Senchou et al. 2004). In many of the RXLR-dEER effectors, the C-terminal domain consists of a variable number of motifs that occur in a repeated fashion (Jiang et al. 2008). This allows rapid evolution and diversification within this effector family, which is consistent with a role for *Avr* genes in gene-for-gene interactions with their hosts.

A wealth of *R* genes is present in botanical *Solanum* species, but in spite of that, resistance breeding has thus far been unsuccessful. Introgressed *R* genes from *S. demissum* and *S. berthaultii*, for example, were quickly defeated and virulent *P. infestans* isolates were detected in the field, sometimes even before introduction into cultivated potato (Flier et al. 2003; Grünwald et al. 2001; Rauscher et al. 2006; Wastie 1991). Recently, renewed hope for resistance breeding emerged with the identification of the so-called 'broad-spectrum' *R* genes *Rpi-blb1* and *Rpi-blb2* from *S. bulbocastanum* (Song et al. 2003; van der Vossen et al. 2003, 2005). Since the introduction in potato, *Rpi-blb1* appears to have remained effective in various geographical areas and over several growing seasons (Colton et al. 2006; Helgeson et al. 1998; Naess et al. 2000). The identification of *ipiO* as the candidate for *Avr-blb1* enables us to address the question of how widespread avirulent alleles or variants occur in *P. infestans* populations and, related to this, how likely it is that *Rpi-blb1*–mediated resistance will last in the field. In this study, we monitored the genetic variation of *ipiO* in a highly diverse set of *P. infestans* isolates and related *Phytophthora* species, and identified 16 naturally occurring *ipiO* variants that could be grouped in three different classes. The *P. infestans* isolates lacking one specific class of *ipiO* variants appeared to be virulent on plants carrying *Rpi-blb1*, thus confirming the gene-for-gene interaction between *ipiO* and *Rpi-blb1*. We also show that IPI-O contains a W motif in the C-terminal domain that is subject to positive selection and that this domain is sufficient to trigger *Rpi-blb1*–dependent cell death.

RESULTS

The *ipiO* gene is highly diverse.

For assessing natural genetic variation of *ipiO*, we compiled a set of 29 *P. infestans* isolates that were collected from various geographic regions, including the European potato-growing areas and the Central Highlands of Mexico that are both known to harbor genetically highly diverse *P. infestans* populations (Flier 2001; Flier et al. 2003; Rivera-Peña 1990a) (Supplementary Table 1). Furthermore, the *P. infestans* isolates originated from diverse host plants, including potato, tomato and botanical *Solanum* species, and they vary for year of collection, mating type, and virulence pattern on the potato *R1-R11* differential set. To examine the genetic diversity, we fingerprinted 21 *P. infestans* isolates with simple sequence repeat (SSR) markers and showed that nearly all isolates had a unique genotype. Only two isolates, i.e., IPO-0 and UK7824, had identical genotypes, and PRC505705 and PRC506303 have a highly similar genotype. No clear supported branching was observed upon neighbor joining in the cluster analysis, illustrating that the assembled set of *P. infestans* isolates is genetically highly diverse (Supplementary Fig. 1).

To determine the genetic variation at the *ipiO* loci, *ipiO* was amplified using polymerase chain reaction (PCR) on genomic DNA derived from the 29 *P. infestans* isolates and from five isolates of other clade 1c species, i.e., *P. andina*, *P. ipomoeae*, *P. phaseoli*, and *P. mirabilis* (Blair et al. 2008). Sequence analyses revealed 16 variants of *ipiO*, with a minimum of one and a maximum of four *ipiO* variants per isolate (Table 1, Fig. 1, Supplementary Fig. 2). So far, no *ipiO* homologs have been detected in *Phytophthora* species outside clade 1c (data not shown). As described previously, IPI-O1 and IPI-O2 are highly similar, with only four different amino acids (Pieterse et al. 1994). Most of the newly identified IPI-O variants have amino-acid changes due to point mutations, i.e., IPI-O3 to IPI-O9, IPI-O11, IPI-Om1, and IPI-Om2. IPI-O10 is identical to IPI-O2, although this variant contains two nonsynonymous nucleotide polymorphisms. Another variant, IPI-O13, has a C-terminal extension of 10 amino acids compared with IPI-O3. *P. infestans* variant IPI-O12 and *P. phaseoli* IPI-Op1 have frameshift mutations that result in truncated proteins.

Phylogenetic analyses on the protein alignment of the 16 IPI-O variants showed a grouping into three classes, designated as classes I, II, and III (Fig. 2). Phylogenetic trees based on neighbor-joining, minimal evolution, or maximum parsimony algorithms resulted in similar clustering (not shown), and analyses based on nucleotide alignments also yielded comparable results. Most IPI-O variants group in class I together with IPI-O1 and IPI-O2. Class II is significantly different from class I and includes IPI-O3 and IPI-O13. Class III comprises IPI-O4, which has 16 amino-acid differences compared with class I and II IPI-O variants and is the most divergent IPI-O variant in *P. infestans*.

IPI-O has one W motif with several positively selected sites.

The majority of the RXLR-dEER effectors contain positively selected amino-acid residues in the C-terminus (Jiang et al. 2008; Win et al. 2007). To investigate whether *ipiO* is also under diversifying selection, we assessed positive selection per residue on two sets of *ipiO* variants. The first set, called Pi, is composed of the 11 full-length *ipiO* variants as found in *P. infestans.* The other set, Pi+Pm, includes the variants detected in the sibling species *P. mirabilis* in addition to the Pi set. Of the different evolutionary models (Yang et al. 2005), model M2a for positive selection fits well on both data sets with ℓ values of –822.55 and –960.76 for Pi and Pi+Pm, respectively. With this model, several positively selected amino-acid residues were identified, four of which overlap in the two data sets. Also the selection model M8 gave high log likelihood values for both sets. For the Pi set, M2a and M8 identified the same seven positively selected sites. For the Pi+Pm set, M8 identified the same set of four overlapping positively selected sites and four additional ones, one of which is also selected by the M2a model for the Pi+Pm set (Table 2).

In a recent study that used hidden Markov model (HMM) searches to find motifs in RXLR-dEER effectors, it was shown that many of the RXLR-dEER effectors contain conserved C-terminal motifs that may occur in repeated fashion (Jiang et al. 2008). These motifs were named W, Y, and L after the amino acid at a fixed position in each motif. IPI-O contains a single W motif with moderate to strong HMM scores ranging from 6.5 to 12.3 among the IPI-O variants (Fig. 1, Supplementary Fig. 3). Interestingly, three of the four positively selected sites that overlap in the M2a and M8 model of the two sets have a high posterior probability ($P > 99\%$) and are located within the conserved W motif (Fig. 1).

The region comprising the W motif is sufficient to trigger *Rpi-blb1*–mediated cell death.

To investigate whether or not the W motif is involved in triggering *Rpi-blb1*–mediated cell death, we analyzed several deletion mutants of *ipiO2* (Fig. 3A). In agro-coinfiltration assays in *N. benthamiana*, IPI-O2—with or without its signal peptide—triggers cell death in the presence of Rpi-blb1. Deleting the domain comprising the RXLR, RGD, and dEER motifs did not abolish recognition, and even an additional deletion of the first 26 amino acids of the C-terminal domain did not change the cell-death response. Agroinfection and rub-inoculation assays on *S. stoloniferum* accession sto17605-4, which harbors the *Rpi-blb1* homolog *Rpi-sto1*, resulted in similar responses and showed that recognition of IPI-O by *Rpi-sto1* follows the same pattern. The results show that the region spanning the last 54 amino acids of IPI-O and comprising the W motif is sufficient for recognition by Rpi-blb1 and Rpi-sto1. Since the only mutations that are consistent between, on the one hand, the class I and II variants and, on the other hand, the class III variant are located within the W motif, it is conceivable that this motif plays a role in recognition of IPI-O by Rpi-blb1.

IPI-O variants of classes I and II but not class III trigger *Rpi-blb1*–mediated cell death.

In a previous study, we showed that the class I *ipiO* variants, *ipiO1* and *ipiO2*, trigger *Rpi-blb1*–mediated cell death. To assess whether the newly identified *ipiO* variants are also recognized by *Rpi-blb1*, we used agroinfiltration in *N. benthamiana* to reconstruct the interaction between the *ipiO* variants and *Rpi-blb1*. Coinfiltration of *N. benthamiana* leaves with an *Agrobacterium tumefaciens* strain carrying a construct expressing *Rpi-blb1* as well as a strain carrying a construct expressing either class I *ipiO* genes (*ipiO1*, *ipiO2*, *ipiO5*, *ipiO7*, and *ipiO8*) or class II *ipiO* genes (*ipiO3*) resulted in a confluent cell-death response (Fig. 3B). Also class I *ipiO* variant *ipiOm2* of *P. mirabilis* coinfiltrated with *Rpi-blb1* resulted in cell death (data not shown). In contrast, coexpression of *Rpi-blb1* with the class III *ipiO4* gene did not elicit a *Rpi-blb1*–mediated response, as no visible cell death was observed in the infiltrated leaves.

IpiO variants are expressed in planta.

To enable expression analyses of *ipiO* variants belonging to the three classes, class-specific primers were designed that were tested for specificity on genomic DNA (Fig. 4). Subsequently, RNA isolated from potato leaves infected with *P. infestans* isolates PIC99183, PIC99189, and PIC99177 was analyzed by semiquantitative reverse transcription (RT)-PCR. As shown in Figure 4, mRNA derived from class I, II, and III *ipiO*

Table 1. Occurrence of *ipiO* variants and classes in isolates of *Phytophthora infestans* and related species

		Class I									II				III		
***Phytophthora* spp.**	**Isolate**	***ipiO1***	***ipiO2***	***ipiO5***	***ipiO6***	***ipiO7***	***ipiO8***	***ipiO9***	***ipiO10***	***ipiO11***	***ipiO12***	***ipiOm2***	***ipiO3***	***ipiO13***	***ipiO4***	***ipiOm1***	***ipiOp1***
P. infestans	F95573	X	X														
	89148-09	X															
	PIC99177												X				
	88069	X	X										X				
	PIC99189												X		X		
	90128	X	X							X							
	EC1	X	X							X			X				
	H30P04	X								X			X				
	USA618	X	X														
	IPO-0	X													X		
	IPO-C	X											X				
	PIC99183									X	X						
	NL01096	X	X										X				
	VK98014	X	X											X			
	IPO428-2	X	X							X							
	NL00228	X	X											X			
	DDR7704		X														
	UK7824		X												X		
	89094			X			X	X					X				
	91011	X			X	X			X								
	PIC97757	X			X	X			X								
	IPO98014			X			X	X					X				
	NL050105		X										X				
	NL05194		X										X				
	PRC505705		X														
	PRC506303		X														
P. infestans s.l.[a]	EC3260														X		
	EC3394														X		
	EC3364	X	X										X				
P. andina	EC3414														X		
P. ipomoeae	PIC99193											X					
P. phaseoli	CBS556.88																X
P. mirabilis	PIC99111											X					
	CBS150.88															X	

[a] s.l. = sensu lato.

genes is present, demonstrating that all three classes comprise functional genes that are expressed during in planta growth.

Isolates lacking class I *ipiO* variants are virulent on *Rpi-blb1* plants.

To test whether the identified *ipiO* variants determine *Rpi-blb1*–mediated cell death, we performed infection assays using *P. infestans* isolates that are genetically diverse and carry different classes of *ipiO* variants (Fig. 1, Table 1). To allow a correct interpretation of the virulence phenotypes of the isolates on *Rpi-blb1* plants, we first tested 16 selected isolates for their infection capabilities on potato. Detached leaves of universal susceptible potato cultivar Désirée were inoculated and, at 6 days postinoculation (dpi), lesion diameters were measured. Based on lesion size (LS), the isolates were grouped in three classes of aggressiveness (Supplementary Table 3). To investigate the specificity spectrum of *Rpi-blb1*, we inoculated the 16 isolates on the *S. bulbocastanum* accession blb8005-8, which is the genotype from which *Rpi-blb1* was isolated, and included Désirée and *S. bulbocastanum* blb2002—containing *Rpi-blb2*—as susceptible and resistant controls, respectively (Table 3). Lesion diameters were measured at 4, 5, and 6 dpi, and LS, lesion growth rates (LGR), and infection efficiency (IE) were calculated. Large lesions exceeding 25 mm^2 always coincided with massive sporulation and were scored as compatible interactions. In contrast, smaller lesions typically did not sporulate or showed a 'black-spot' phenotype, indicating a HR. As expected for the so-called broad-spectrum *R* gene *Rpi-blb1*, blb8005-8 was incompatible with nearly all isolates. Two Mexican isolates, PIC99177 and PIC99189, however, were clearly compatible with blb8005-8 and both developed sporulating lesions on blb8005-8 leaves.

To investigate the correlation between compatibility or incompatibility and *ipiO* variants, we compared the virulence phenotypes of the isolates on *Rpi-blb1*–containing host plants with the occurrence or the absence of specific *ipiO* variants. All avirulent isolates contained at least one class I *ipiO* variant (Tables 1 and 3). In contrast, no class I *ipiO* variants were found in the two virulent isolates PIC99189 and PIC99177; only class II and III *ipiO* variants were detected. These results suggest that class I *ipiO* variants determine avirulence of *P. infestans* isolates on *Rpi-blb1* plants.

To verify these findings, we tested the *P. infestans* isolates on transgenic lines of cultivar Impala and cultivar Désirée containing *Rpi-blb1* as transgene. In general, *Rpi-blb1*–meditated resistance levels in the potato transgenic lines were lower than in its wild *Solanum* background, blb8005-8 (Table 3). In accordance with the previous experiment, isolate PIC99189 was able to establish sporulating lesions on transgenic Impala RGC-2A9 expressing *Rpi-blb1*, whereas isolates IPO-C and 90128 displayed a HR (Fig. 5). On five Désirée *Rpi-blb1* transformants, including A01-20, we quantitatively assessed the resistance levels. The virulent isolates PIC99177 and PIC99189 infected A01-20 (Table 3) and the other four transgenic lines (data not shown) equally well as the Désirée control plants (analysis of variance, $P < 0.05$). To the other 14 isolates, the *Rpi-blb1* transgene conferred enhanced resistance at various levels, and generally, the level of resistance negatively correlated with the aggressiveness of the isolates. The moderately aggressive isolates F95573, 89148-09, and 88069 reached only low levels of IE and LGR on A01-20, and *Rpi-blb1* clearly conferred a high level of resistance. Highly aggressive isolates, however, achieved slightly reduced or similar IE and LGR on A01-20 as compared with Désirée control plants and were often able to establish high percentages of fast growing lesions on A01-20, despite the fact that these isolates contain a class I *ipiO* variant. Obviously, the aggressiveness of the isolates overrules the recognition by *Rpi-blb1* in the transgenic potato background. The observation that the level of resistance conferred by *R* genes is influenced by the genetic background in

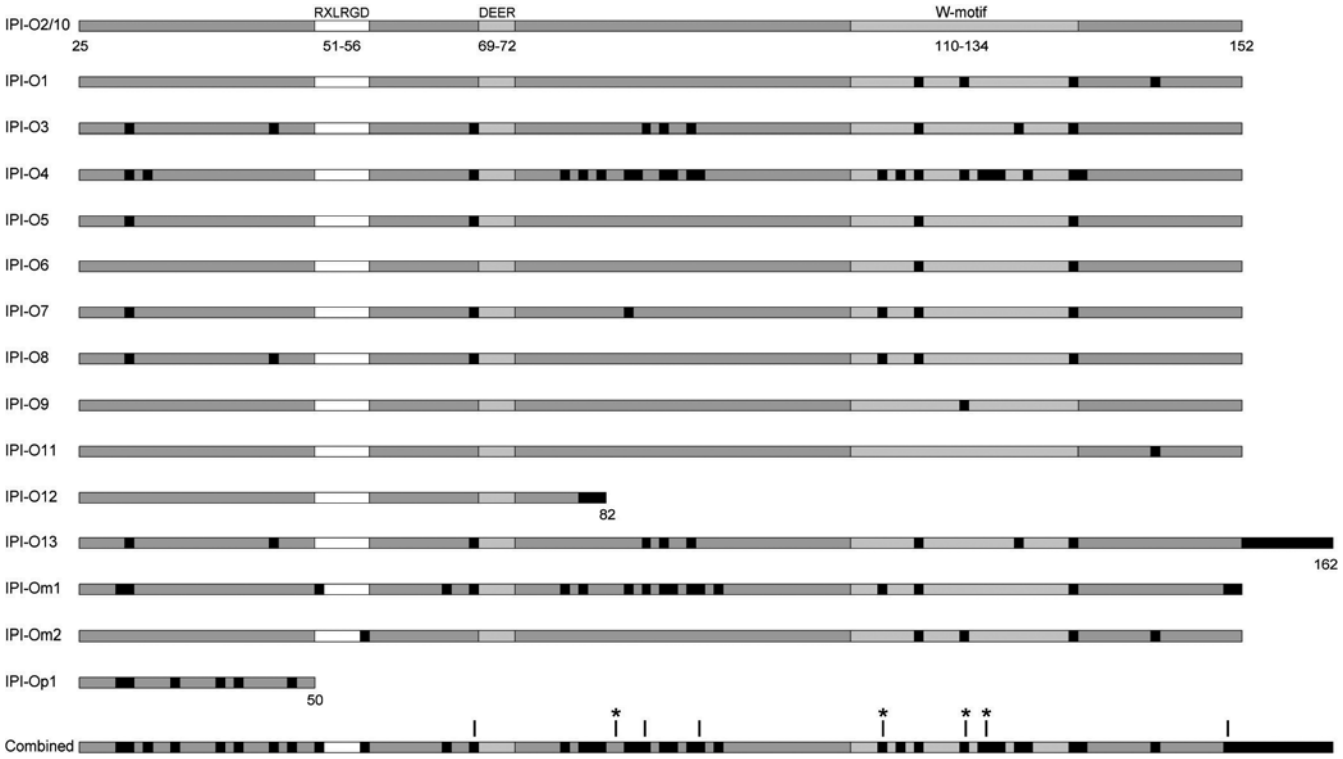

Fig. 1. Thirteen variants of the mature IPI-O protein. The motifs RXLR, RGD, and DEER and a predicted W motif are indicated. Numbers refer to the amino-acid positions. Amino-acid polymorphisms in IPI-O variants, as compared with IPI-O2, are depicted in black. In the lower bar (combined), all the amino acids that show polymorphism are indicated. The vertical lines refer to positively selected sites. * = $P < 99\%$.

which they reside is in line with previous studies. For example, it was shown that expression of *Rpi-blb1* in *S. bulbocastanum* is dramatically higher than in potato lines carrying *Rpi-blb1* as a transgene (Bradeen et al. 2009; Kramer et al. 2009).

Recognition specificity of *ipiO* variants by *Rpi-blb1* is conserved in *Rpi-sto1* and *Rpi-pta1*.

Recently, we described the identification and cloning of functional homologs of *Rpi-blb1* in the distantly related *Solanum* species *S. stoloniferum* (Vleeshouwers et al. 2008). The homologs *Rpi-sto1* and *Rpi-pta1* are nearly identical to *Rpi-blb1*; they only differ in three and five nonsynonymous nucleotide substitutions, respectively. To test these *R* genes for *ipiO* specificity, we coinfiltrated *N. benthamiana* leaves with *A. tumefaciens* strains carrying constructs expressing either *Rpi-sto1* or *Rpi-pta1* combined with *A. tumefaciens* strains expressing *ipiO* variants of class I, II, or III. Leaves coinfiltrated with the *Rpi-blb1* homologs and class I or class II *ipiO* variants showed a confluent cell-death response, but leaf panels coinfiltrated with class III *ipiO* did not show cell death (Supplementary Fig. 4). These results demonstrate that, similar to *Rpi-blb1*, *Rpi-sto1* or *Rpi-pta1* display differential specificity towards the different classes of *ipiO* variants.

We also investigated how *S. stoloniferum* sto17605-4 and pta17831-8 responded to infection with the 16 *P. infestans* isolates that posses different variants of *ipiO*. In detached leaf assays, infection of sto17605-4 and pta17831-8 with the isolates PIC99189 and PIC99177 resulted in sporulating lesions, in accordance with the results obtained with blb8005-8. Of the other 14 isolates, only PIC99183 formed a few lesions on sto17605-4 and pta17831-8. There was, however, no biotrophic growth nor sporulation and the lesions expanded very slowly. Since the LGR and IE parameters were much lower than expected for such a highly aggressive isolate, we suspect that the necrotic spots are due to a trailing HR following invasion attempts but not to specific virulence of PIC99183 on *S. stoloniferum* sto17605-4 and pta17831-8.

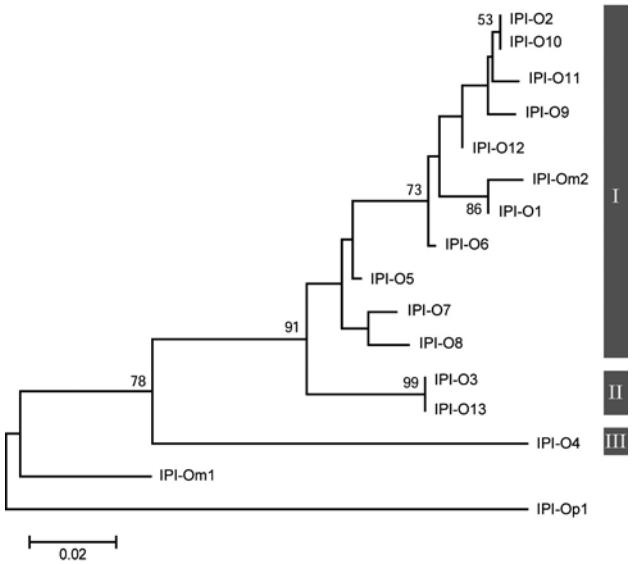

Fig. 2. Phylogenetic relationship and clustering of the IPI-O variants. The minimum evolution tree was rooted with IPI-Op1. Rooting with IPI-Om1, IPI-O4, or *Phytophthora sojae* Avr1b resulted in similar clustering. Bootstrap values of 1,000 replicates are indicated at the nodes; values less than 50% are omitted (Felsenstein 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. Class I, II, and III IPI-O variants are indicated.

DISCUSSION

Rpi-blb1 is classified as a so-called broad-spectrum *R* gene that confers resistance to a broad range of *P. infestans* isolates (Song et al. 2003; van der Vossen et al. 2003). In a recent study, we identified the RXLR-dEER effector IPI-O as the candidate for the cognate Avr factor of Rpi-blb1 (Vleeshouwers et al. 2008). In this study, we detected isolates that are virulent on Rpi-blb1 plants and showed that *Rpi-blb1–ipiO* is an *R-Avr* pair that basically interacts according to the gene-for-gene model (Flor 1971). IPI-O is the effector that triggers ETI in plants carrying *Rpi-blb1*, whereas changes in the IPI-O effector repertoire result in loss of ETI.

The *ipiO* gene family is highly diverse but restricted to *Phytophthora* species that belong to clade 1c. Profiling 34 isolates of *P. infestans* and its close relatives revealed 16 *ipiO* variants, 14 of which were grouped in three distinct classes. In *P. infestans* sensu latu, *P. andina*, *P. ipomoeae*, *P. phaseoli*, and *P. mirabilis*, which all evolved on host species distant from *Solanum* section Petota, a more or less equal distribution of class I, II, and III variants was found, and in addition, two more distant *ipiO* variants were detected. In *P. infestans* however, class I was significantly expanded, with one to four class I variants in most isolates, of which the majority also has one class II or one class III variant or both. Class I includes *ipiO1* and *ipiO2*, the variants that were already isolated in the early 1990s (Pieterse et al. 1994) and, more recently, were identified as *Avr-*

Table 2. Evidence for positively selected sites in *ipiO*

Model code	Parameter estimates	ℓ[a]	Positively diversified codons[b]
Pi[c]			
M0: one ratio	$\omega = 1.089$	–839.78	Not allowed
M1a: nearly neutral	$\omega0 = 0$, $\omega1 = 1$, $p0 = 0.580$, $p1 = 0.420$	–835.50	Not allowed
M2a: positive selection	$\omega0 = 0$, $\omega1 = 1$, $\omega2 = 28.638$, $p0 = 0.353$, $p1 = 0.637$, $p2 = 0.010$	**–822.55**	46N, 82Y, 85M, 87L, 113A*, 122R*, 124L*
M7: beta	$p = 0.012$, $q = 0.005$	–837.03	Not allowed
M8: beta and ω	$p0 = 0.990$, $p1 = 0.010$, $p = 0.008$, $q = 0.005$, $\omega = 27.108$	**–822.57**	46N, 82Y, 85M, 87L, 113A*, 122R*, 124L*
Pi + Pm[d]			
M0: one ratio	$\omega= 0.970$	–980.05	Not allowed
M1a: nearly neutral	$\omega0 = 0$, $\omega1 = 1$, $p0 = 0.599$, $p1 = 0.401$	–972.94	Not allowed
M2a: positive selection	$\omega0 = 0.476$, $\omega1 = 1$, $\omega2 = 12.487$, $p0 = 0.971$, $p1 = 0$, $p2=0.028$	**–960.76**	85M*, 93G, 113A*, 122R*, 124L
M7: beta	$p = 0.005$, $q = 0.007$	–972.94	Not allowed
M8: beta and ω	$p0 = 0.983$, $p1 = 0.017$, $p = 0.005$, $q = 0.005$, $\omega = 14.665$	**–960.34**	68S, 85M*, 87L, 93G, 113A*, 122R*, 124L*, 151P

[a] Log likelihood value.
[b] Bayes Empirical Bayes analysis (Yang et al. 2005). Positively selected sites ($P > 95\%$, * = $P > 99\%$).
[c] Based on full-length *P. infestans ipiO* variants, $n = 11$.
[d] Based on full-length *P. infestans* and *P. mirabilis ipiO* variants, $n = 13$.

blb1 (Vleeshouwers et al. 2008). Disease testing on *Solanum* species showed that all isolates with class I *ipiO* variants were avirulent on *Rpi-blb1* plants, which is in line with the previously reported broad-spectrum character of this *R* gene (Song et al. 2003; van der Vossen et al. 2003). In contrast, two Mexican *P. infestans* isolates, PIC99189 and PIC99177, each of which lack class I *ipiO* variants, appeared to be virulent on *Rpi-blb1* plants. These data suggest that absence of class I *ipiO* genes is correlated with virulence on *Rpi-blb1* plants, and this is supported by the observation that coinfiltration of class I *ipiO* variants and *Rpi-blb1* in *N. benthamiana* leaves results in cell death. Apparently, class I IPI-O variants elicit HR in *Rpi-blb1* plants and this arrests pathogen invasion. Class III *ipiO* appeared unable to induce cell death when coinfiltrated with *Rpi-blb1*, thus strongly suggesting that the presence of class III *ipiO* in *P. infestans* strains is indeed unlikely to confer avirulence. It should be noted, though, that we have not used epitope-tagged constructs in our in planta expression assays to monitor the stability of the various IPI-O variants and, hence, cannot exclude the possibility that class III IPI-O is less stable than class I or II IPI-O. A more puzzling issue is the finding that class II variants elicit cell death when coinfiltrated with *Rpi-blb1* in *N. benthamiana* leaves. The class II variant *ipiO3* was found to be expressed in planta in the virulent strain, and one would expect that the presence of a class II variant together with the Rpi-blb1 resistance protein in one cell would lead to HR. This is not the case and raises the question how the numerous RXLR effectors that are predicted to be targeted to the host cell interact with each other. In reconstruction experiments, for example, some RXLR effectors suppress cell death induced by other effectors such as elicitins or BAX (Bos et al. 2009; Bouwmeester et al. 2008; Dou et al. 2008). In vivo, there could well be a kind of synergism between class I and class II IPI-O variants or even other RXLR effectors. For example, when class I is lacking, the class II variant might not be potent enough to act as Avr factor by itself. Alternatively, the presence of a class I variant could suppress the virulence function of a class II variant by competition for the same virulence target. One

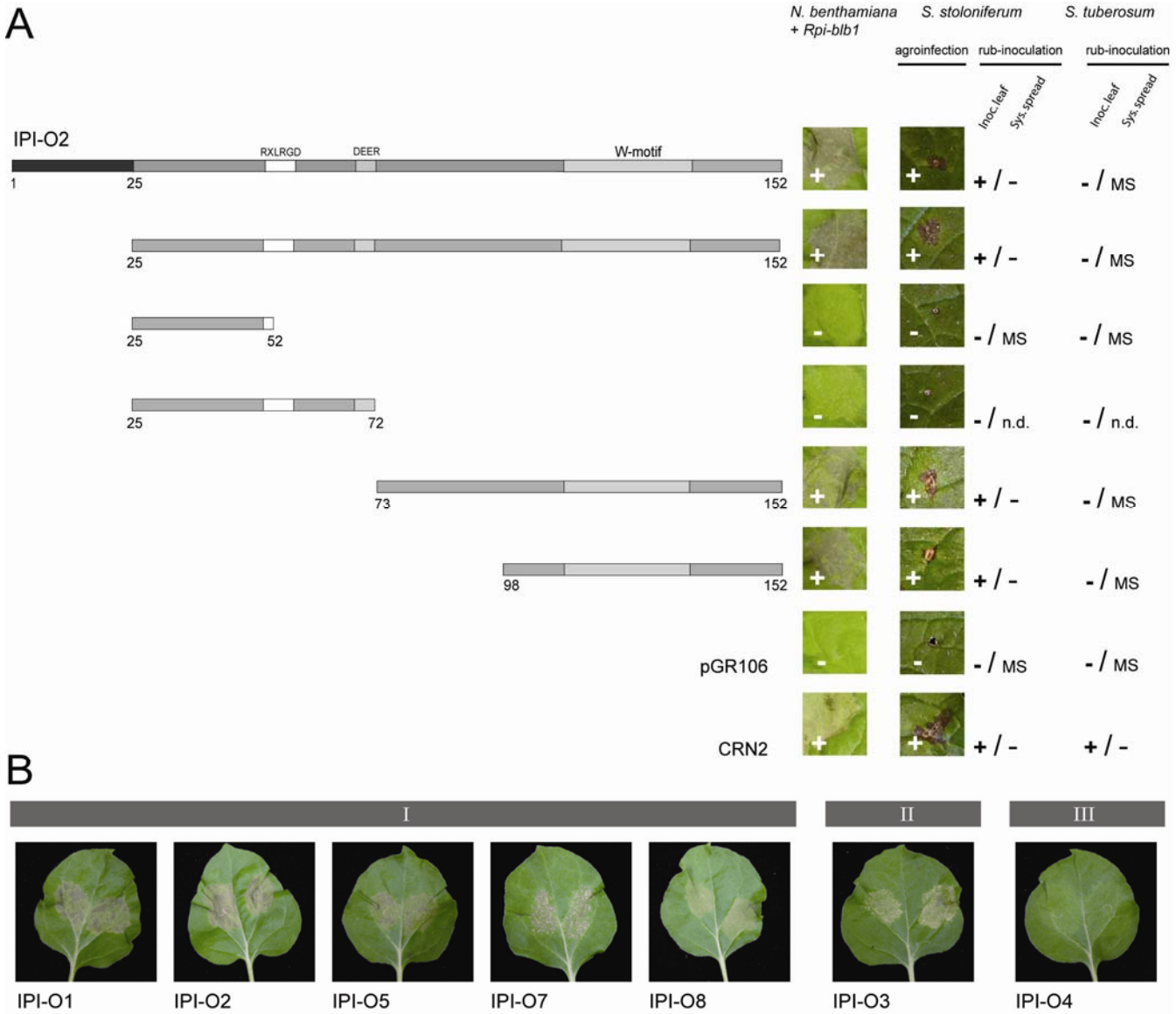

Fig. 3. A, The C terminus of IPI-O comprising the W motif is sufficient for recognition by Rpi-blb1. Deletion mutants of *ipiO*2 were agro-coinfiltrated with *Rpi-blb1* in *Nicotiana benthamiana* or were agroinfected or rub-inoculated with *Potato virus X* particles carrying the deletion mutants on *Solanum stoloniferum* accession 17605-4. Potato cultivar Bintje was used as control. Mosaic symptoms (MS) indicate virus spread. Pictures were taken at 5 days after infiltration or agroinfection. **B,** Class I and II IPI-O variants are recognized by Rpi-blb1. *N. benthamiana* leaves were agroinfiltrated at both sides of the leaf midrib with *Rpi-blb1* in combination with *ipiO* variants of classes I, II, or III. Pictures were taken at 5 days after infiltration.

can also not rule out the possibility that the class II variants are less stable in planta than the class I variants. The finding of a class III variant that is not recognized by *Rpi-blb1* but is expressed in planta makes the situation even more complex.

Nevertheless, the clear distinction between the three classes helped us to define which parts of the protein are involved in recognition by Rpi-blb1. Deletion analysis showed that the C-terminal part of the IPI-O effector protein is required for recognition, which is in line with other studies (Bos et al. 2006; Dou et al. 2008). The smallest fragment that we tested is 54 amino acids in length and comprises the single W motif that is present in IPI-O. For *Phytophthora sojae* Avr1b, specific amino-acid residues in the W- and Y-motifs in Avr1b are responsible for recognition by the RPS1 protein as well as suppressor activity of cell death. In IPI-O, the W motif is the region that is the most divergent in the class III variants, and the three amino acids that show positive selection are all located within the W motif. More detailed analysis of the role of each individual amino-acid residue in the W motif will reveal the exact determinants of recognition by Rpi-blb1.

In recent years, insights into *R* gene–based resistance in potato and the role of cognate *Avr* genes from *P. infestans* have increased. For example, many studies were performed for the *R3a-Avr3a* model system. The *R3a*-harboring species *S. demissum* coexists with *P. infestans* in the cool and humid mountain forests in Toluca Valley, a perfect condition for a tight coevolution between *R3a* and *Avr3a*. *R3a* is a typical fast-evolving type I *R* gene (Huang 2005; Kuang et al. 2005), resulting in numerous *R3a*-like genes (Friedman and Baker 2007; Huang 2005). For Avr3a, only two alleles have been detected; $Avr3a^{EM}$ is present in most *P. infestans* isolates world-wide (Armstrong et al. 2005; Rivera-Peña 1990b), whereas $Avr3a^{KI}$ is much less abundant. $Avr3a^{KI}$ but not $Avr3a^{EM}$ is recognized by R3a, thus resulting in defeat of *R3a* by most *P. infestans* isolates. *R1*, *R2*, and *R4* also originate from *S. demissum* and, similar to *R3a*, these *R* genes were quickly defeated in the field (Fry 2008). Similar to *Avr3a*, *Avr4* alleles in field isolates show very little variation and, in all virulent strains, the *Avr4* gene is out of frame, due to a 1 base pair deletion (van Poppel et al. 2008). Preliminary analysis of *Avr1* and *Avr2* indicates also that these *Avr* genes are represented by only a few alleles (F. Govers, P. Birch, and E. Gilroy, *personal communication*). A completely different scenario exists for the *Rpi-blb–ipiO* interaction. *Rpi-blb1* originates from *S. bulbocastanum*, which occurs in more arid climates and most likely has less intensive encounters with *P. infestans.* Presence of *Rpi-blb1* homologs in other Mexican species such as *S. stoloniferum* (Wang et al. 2008) that partly grow in *P. infestans* conducive climates creates the opportunity for virulent strains to evolve on these species. Indeed, the virulent strains PIC99189 and PIC99177 described in this study were collected from *S. stoloniferum* host plants in Mexico (Flier et al. 2002). Both *Rpi-sto1* and *Rpi-pta1* are almost identical to *Rpi-blb1*, and *Rpi-blb1* fulfills the criteria of a type II *R* gene with only little diversifying selection that is typically slow evolving (Kuang et al. 2005; Liu and Halterman 2006). Thus, for the *Rpi-blb1–ipiO* interaction, not the *R* gene but the *Avr* gene is represented by a highly diverse and extensive gene family. The notable expansion of class I *ipiO* in *P. infestans* but not in related *Phytophthora* species that infect other plant species might be due to a certain degree of coevolution between *Avr-blb1* and *Rpi-blb1* homologs in *Solanum* host plants. Also *Rpi-blb2*, another broad-spectrum *S. bulbocastanum R* gene that is not (yet) defeated, is interacting with a highly diverse *Avr* gene family, and perhaps such *Avr* genes may be less easy to overcome (S. Kamoun, *personal communication*).

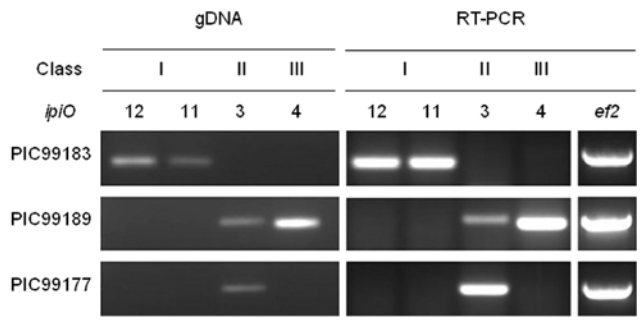

Fig. 4. Expression of *ipiO* in infected potato leaves. *IpiO* class-specific primers were used for semiquantitative reverse transcription-polymerase chain reactino on RNA isolated from infected leaf tissue of potato cultivar Bintje at 6 days postinoculation. To demonstrate that the primers are specific for each of the three *ipiO* classes, genomic DNA of *Phytophthora infestans* isolates PIC99183, PIC99189, and PIC99177 was used as template and detection of specific *ipiO* variants was confirmed. The *P. infestans* elongation factor 2 gene (*ef2*) was used as a control to determine the integrity of the RNA.

Table 3. *Phytophthora infestans* isolates virulent on *Solanum* plants containing *Rpi-blb1* lack class I *ipiO* variants

	Plant material													*ipiO* class		
	blb8005-8		sto17605-4		pta17831-8		A01-20		Désirée		blb2002					
***P. infestans* isolate**[a]	**IE**[b]	**LGR**[b]	**IE**	**LGR**	**IE**	**LGR**	**IE**	**LGR**	**IE**	**LGR**	**IE**	**LGR**	*[c]	**I**	**II**	**III**
F95573	0	0	0	0	0	0	3	1.4	69	4.4	0	0	A	X		
89148-09	0	0	0	0	0	0	16	1.1	69	2.7	0	0	A	X		
88069	0	0	0	0	0	0	22	0.6	100	3.2	0	0	A	X	X	
90128	0	0	0	0	0	0	50	3.7	78	3.4	0	0	A	X		
EC1	0	0	0	0	0	0	31	2.5	84	3.7	0	0	A	X	X	
IPO-0	0	0	0	0	0	0	78	3.4	91	3.8	0	0	A	X		X
PIC99183	0	0	50	0.5	63	1.2	88	3.6	91	3.6	0	0	A	X		
H30P04	0	0	0	0	0	0	9	2.4	100	4.4	0	0	A	X	X	
IPO-C	0	0	0	0	0	0	50	2.9	100	3.9	0	0	A	X	X	
USA618	0	0	0	0	0	0	19	3.5	100	3.4	0	0	A	X		
NL01096	0	0	0	0	0	0	66	3.4	100	3.8	0	0	A	X	X	
VK98014	0	0	0	0	0	0	66	1.8	100	4.7	0	0	A	X	X	
IPO428-2	0	0	0	0	0	0	38	1.9	100	4.2	0	0	A	X		
NL00228	0	0	0	0	0	0	47	4.6	100	4.8	0	0	A	X	X	
PIC99177	72	1.1	75	2.6	81	2.9	50	2.8	88	3.0	0	0	V		X	
PIC99189	34	1.1	75	0.9	100	4.3	91	4.5	91	3.6	0	0	V		X	X

[a] Additional information about the aggressiveness of the *P. infestans* isolates on potato cultivar Désirée can be found in Supplementary Table 3.
[b] Isolates were inoculated on different *Solanum* plants containing *Rpi-blb1* or its homologs and mean infection efficiency (IE) and lesion growth rate (LGR) were determined.
[c] *Phenotype on *Rpi-blb1* plants. A = avirulent; V = virulent.

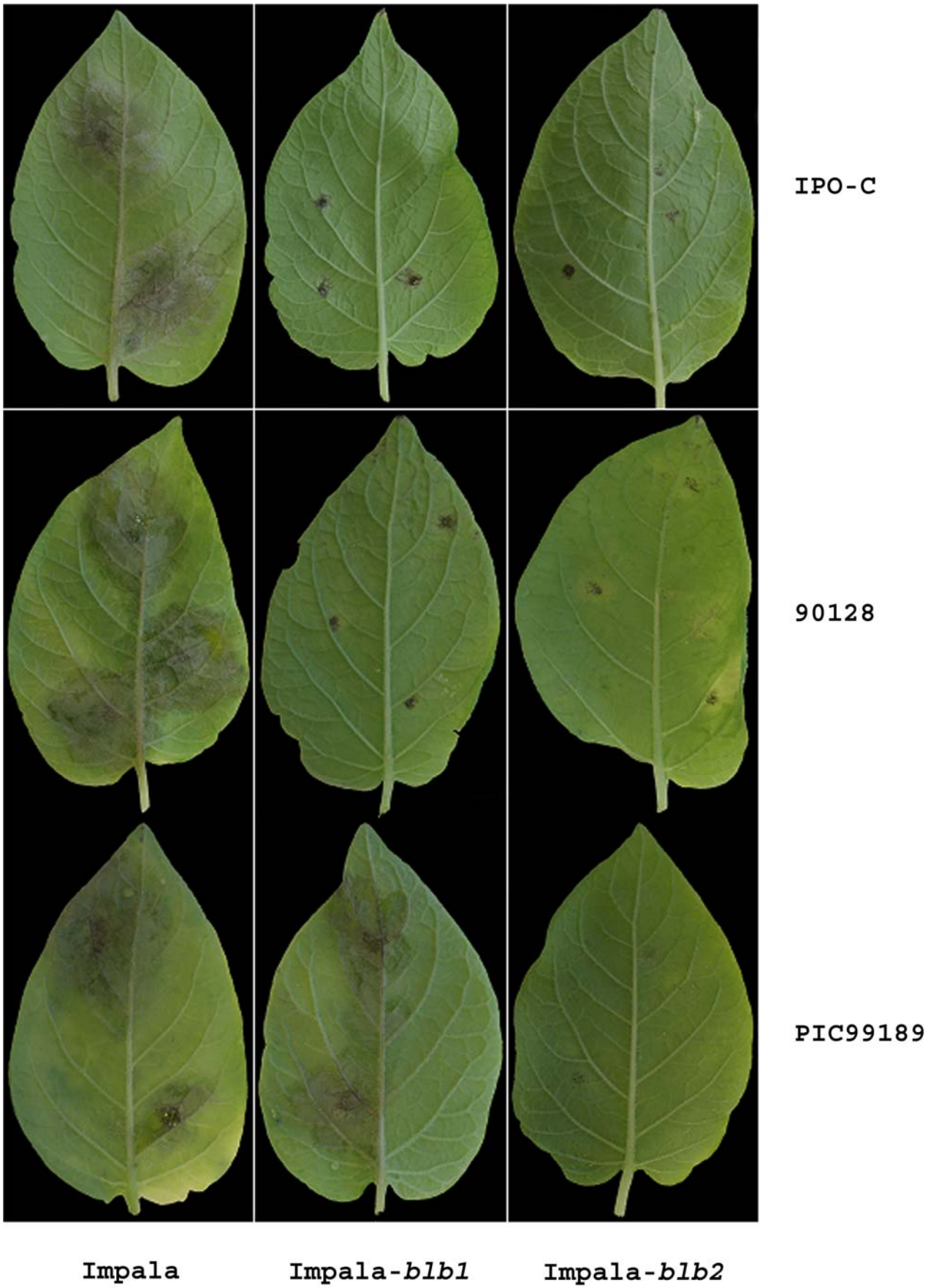

Fig. 5. *Rpi-blb1* transgenic potato lines are resistant to *Phytophthora infestans* isolates carrying *ipiO* class I variants, class II variants, or both. Pictures were taken at 6 days postinoculation.

In the wild *Solanum* species that contain *Rpi-blb1* or a functional homolog, full resistance was conferred to all tested avirulent *P. infestans* isolates, with the exception of the two Mexican strains. The transfer of *Rpi-blb1* into potato cultivars resulted in improved protection to *P. infestans* isolates. The enhanced resistance, however, could only arrest the growth of mild isolates. Aggressive isolates were not blocked and could still cause high percentages of lesions expanding at high rates. The influence of the genetic background on the performance of *Rpi-blb1* was also reported in other studies, in which the basal *Rpi-blb1* expression levels were found dramatically higher in its wild *S. bulbocastanum* origin compared with transgenic potato (Bradeen et al. 2009; Kramer et al. 2009). Also the increase in expression after *P. infestans* inoculation was higher. These studies suggest that expression of *Rpi-blb1* as transgene in potato is not high enough to provide satisfactory resistance in the field, and this implies that stacking with other *R* genes or engineering enhanced transgene expression in cultivars is recommended.

The future of late blight resistance breeding is controlled application of well-studied *R* genes in high quality potato cultivars and devising inherent durability predictions based on the interacting *Avr* gene. Potato cultivars engineered with *Rpi-blb1* and *Rpi-blb2* are expected to be the first genetically modified potatoes to be cultivated for consumption purposes in Europe (Application GM field trial 2005). *Rpi-blb1* is still effective to a broad range of isolates; virulent isolates similar to PIC99189 and PIC99177 have not (yet) been detected in The Netherlands and neighboring countries, and class I *ipiO* is well-represented in the *P. infestans* isolates analyzed thus far. Future large-scale monitoring aimed at diagnosing the *ipiO* classes in the *P. infestans* population can help determine whether virulence to *Rpi-blb1* is evolving in commercial potato growing areas or whether accidental introduction of potentially virulent *P. infestans* has occurred. When the first virulent isolates are detected, selection pressure towards losing class I *ipiO* might be avoided by omitting cultivars with *Rpi-blb1* for a certain period of time and applying other *R* genes instead.

MATERIALS AND METHODS

Phytophthora isolates, culture conditions, and inoculum preparation.

The *Phytophthora* isolates used in this study were retrieved from our in-house collection or were provided by colleagues. *Phytophthora* isolates were routinely grown in the dark at 15°C in liquid Plich medium (van der Lee et al. 1997) prior to DNA extraction (Lees et al. 2006) or on solid rye sucrose medium (Caten and Jinks 1968) prior to disease tests. To isolate zoospores for plant inoculations, sporulating mycelium was flooded with cold water and incubated at 4°C for 1 to 3 h.

Cloning of *ipiO* variants.

Primers (Supplementary Table 2) and *Pfu* DNA polymerase (Promega, Charbonnnières, France) were used to amplify *ipiO* on genomic DNA. After 30 cycles, Super*Taq* polymerase (HT Biotechnology, Cambridge) and its buffer were added, followed by 15 min at 72°C. The obtained amplicons were cloned into pGEM-T Easy vector (Promega) and were transformed in DH5α competent cells (Invitrogen, Carlsbad, CA, U.S.A.). Sequencing was performed using universal M13 primers and DNA sequences were analyzed using DNAstar v6, Chromas 2.3 (Technelysium, Tewantin, Australia) and Vector NTI software.

SSR genotyping.

SSR genotyping was performed using two multiplex sets of four SSR markers each (SSR1, SSR3, SSR7, SSR11, and SSR2, SSR4, SSR6, SSR8). Experimental details can be found at the Eucablight website.

Phylogenetic data analyses.

The SSR data were analyzed by the phylogenetic software package TREECON for Windows version 1.3b (van de Peer and de Wachter 1994). The evolutionary distance estimation was performed according to Nei and Li (1979), and clustering was performed using the neighbor-joining algorithm. The tree was rooted using isolate IPO-0. Bootstrap values in percentages (>60) from 1,000 replicate trees are shown at the nodes. The scale bar shows genotype divergence as a percentage. Phylogenetic analyses of ipiO sequences were conducted using the minimum evolution method (Rzhetsky and Nei 1992) in MEGA version 4 (Tamura et al. 2007).

Positive selection analysis.

To test for amino acids under purifying or diversifying selection, we used codon-based analysis (Codeml) implemented in PAML v. 4 package (Yang 2007). Maximum-likelihood codon substitution models M0, M1a, M2a, M7, and M8 were used for analysis. Models M2a and M8 are capable of detecting sites under positive selection. Bayes Empirical Bayes statistics was used to calculate positively selected sites with high posterior probability (Yang et al. 2005).

IpiO expression analysis.

The zones surrounding the water-soaked lesions, in which *ipiO1* is known to be highly expressed (van West et al. 1998), were cut from infected leaves of cultivar Bintje at 6 dpi. RNA was isolated using the RNeasy mini kit (Qiagen, Hilden, Germany), was incubated with Dnase, and was purified with the RNA clean-up protocol. The purity of the RNA was confirmed on gel. Semiquantitative RT-PCR was performed with the OneStep RT-PCR kit (Qiagen), using the specific primers RT-ipiO-I-F and RT-ipiO-I12-R for *ipiO12* (class I), RT-ipiO-I-F and RT-ipiO-I11-R for *ipiO11* (class I), RT-ipiO-II-F and RT-ipiO-II-R for *ipiO3* (class II), and RT-ipiO-III-F and RT-ipiO-III-R for *ipiO4* (class III). *P. infestans* elongation factor 2 gene (*ef2*) was used as a control (Torto et al. 2002).

Plant material and generation of *Rpi-blb1* transgenic potato plants.

Solanum plant material used in this study is listed in Supplementary Table 4. Potato cultivars and wild *Solanum* accessions were obtained from our-in-house collection and the Center of Genetic Resources (CGN), Wageningen, The Netherlands. *Solanum* plants were maintained in vitro on Murashige and Skoog (MS) medium (Duchefa, Haarlem, The Netherlands) supplemented with 20% sucrose (MS20) in climate chambers at 18°C with a 16-h photoperiod. Top shoots were transferred to fresh medium and, 1 to 2 weeks later, rooted plantlets were transferred to the soil and were grown under greenhouse conditions. *N. benthamiana* plants used for agroinfiltration were grown in climate chambers at 22 to 25°C and high light intensity. The generation of the *Rpi-blb1* transformant in cultivar Impala has been described previously (van der Vossen et al. 2003), and the transformant A01-20 of cultivar Désirée was generated using similar procedures. Briefly, the binary vector pBINPLUS containing *Rpi-blb1* under control of its native promoter and terminator (van der Vossen et al. 2003) was transformed to *A. tumefaciens* COR308 and was introduced into cultivar Désirée according to standard protocols (Visser et al. 1991). Regenerative shoots were transferred on solid selective medium Zcvk (MS20 with zeatine at 1 mg liter^{-1}, claforan at 200 mg liter^{-1}, vancomycine at 200 mg liter^{-1}, and kanamycin at 100 mg liter^{-1}) and transformants to solid MS30 with kanamycin at 100 mg liter^{-1}.

Disease tests.

Leaves from 6- to 8-week-old plants were detached and placed in water-saturated oases in trays (Vleeshouwers et al. 1999). Leaves were spot-inoculated at the abaxial leaf side with 10-µl droplets containing 5×10^4 zoospores per milliliter and were incubated in a climate chamber at 15°C with a 16-h photoperiod. Lesion diameters were measured at 4, 5, and 6 dpi. The area of the lesions (LS) and the IE representing the percentage of successful infections were calculated, and the LGR was estimated, using linear regression in GenStat 10.

In planta expression assays.

IpiO variants without signal peptide were introduced in pK7WG2 and pGR106 (Karimi et al. 2002). *R3a*, *Rpi-blb1*, *Rpi-sto1*, and *Rpi-pta1* with their native expression elements were introduced into the pBINPLUS binary vector (van Engelen et al. 1995). *A. tumefaciens* GV3101 and AGL1 (Lazo et al. 1991) in combination with the ternary plasmid pBBR1MCS-5.*virGN54D* (van der Fits et al. 2000) were used for transformation. For agroinfiltration, *A. tumefaciens* strains were grown as described previously (van der Hoorn et al. 2000) to a final optical density at 600 nm of 0.4. Leaves of 4- to 5-week-old *N. benthamiana* plants were infiltrated with the *A. tumefaciens* suspensions (at a 1:1 ratio for coinfiltration) in MMA induction buffer (1 liter of MMA = 5 g of MS salts, 1.95 g of morpholineethanesulfonic acid, 20 g of sucrose, 200 µM acetosyringone, pH 5.6), and responses were scored from 3 to 8 days postinfiltration. To obtain *Potato virus X* (PVX) particles for PVX agroinfection, *Agrobacterium* strains containing the various recombinant pGR106-*ipiO* plasmids were agroinfected on *N. clevelandii* plants. After appearance of mosaic symptoms, leaves were ground in 50 mM potassium phosphate buffer (pH 7.0). Potato plants were rub-inoculated with the obtained homogenate after light dusting with Carborundum powder.

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AUTHOR RECOMMENDED INTERNET RESOURCES

EUCABLIGHT, the potato late blight network for Europe: www.eucablight.org/EucaBlight.asp
Wageningen University Center of Genetic Resources (CGN) website: www.cgn.wur.nl/UK/CGN+Plant+Genetic+Resources/Collections/Potato/-+Species/