In Planta Expression Screens of Phytophthora infestans RXLR Effectors Reveal Diverse Phenotypes, Including Activation of the Solanum bulbocastanum Disease Resistance Protein Rpi-blb2

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The Irish potato famine pathogen Phytophthora infestans is predicted to secrete hundreds of effector proteins. To address the challenge of assigning biological functions to computationally predicted effector genes, we combined allele mining with high-throughput in planta expression. We developed a library of 62 infection-ready P. infestans RXLR effector clones, obtained using primer pairs corresponding to 32 genes and assigned activities to several of these genes. This approach revealed that 16 of the 62 examined effectors cause phenotypes when expressed inside plant cells. Besides the well-studied AVR3a effector, two additional effectors, PexRD8 and PexRD3645-1, suppressed the hypersensitive cell death triggered by the elicitor INF1, another secreted protein of P. infestans. One effector, PexRD2, promoted cell death in Nicotiana benthamiana and other solanaceous plants. Finally, two families of effectors induced hypersensitive cell death specifically in the presence of the Solanum bulbocastanum late blight resistance genes Rpi-blb1 and Rpi-blb2, thereby exhibiting the activities expected for Avrblb1 and Avrblb2. The AVRblb2 family was then studied in more detail and found to be highly variable and under diversifying selection in P. infestans. Structure-function experiments indicated that a 34–amino acid region in the C-terminal half of AVRblb2 is sufficient for triggering Rpi-blb2 hypersensitivity and that a single positively selected AVRblb2 residue is critical for recognition by Rpi-blb2.

INTRODUCTION

Our understanding of the pathogenicity mechanisms of filamentous microbes, such as oomycetes and fungi, has been limited mainly to the development of specialized infection structures, secretion of hydrolytic enzymes, production of host selective toxins, and detoxification of plant antimicrobial compounds (Idnurm and Howlett, 2001; Taibot, 2003; Randall et al., 2005). Recent findings, however, significantly broadened our view of pathogenicity to reveal that filamentous pathogens are much more sophisticated manipulators of plant cells than previously anticipated. Indeed, similar to bacterial pathogens, eukaryotic pathogens secrete an arsenal of proteins, termed effectors, that modulate plant innate immunity and enable parasitic colonization and reproduction (Birch et al., 2006; Chisholm et al., 2006; Kamoun, 2006; O’Connell and Panstruga, 2006; Catanzariti et al., 2007; Kamoun, 2007). Although effectors are thought to function primarily in virulence, they can also elicit innate immunity in plant varieties that carry cognate disease resistance (R) proteins. In such cases, effectors are said to have an avirulence (Avr) activity, thereby activating directly or indirectly programmed cell death (hypersensitive response [HR]) and associated resistance responses mediated by specific R proteins. Deciphering the virulence and avirulence activities of effectors to understand how pathogens interact and coevolve with their host plants has become a driving research paradigm in the field of oomycete and fungal pathology. In particular, the recent availability of genome-wide catalogs of effector secretomes from dozens of filamentous pathogen genome sequences calls for high-throughput approaches (effectomics) to rapidly and efficiently assign functions to computationally predicted effector genes.

The oomycetes form a phylogenetically distinct group of eukaryotic microorganisms that includes some of the most destructive pathogens of plants (Kamoun, 2003). The most
notorious oomycete is the potato (Solanum tuberosum) and
tomato (Solanum lycopersicum) late blight pathogen
Phytophthora infestans. A pathogen of historical significance as the
cause of the Irish potato famine, P. infestans not only continues
to cost modern agriculture billions of dollars annually but also
impacts subsistence farming in developing countries (Kamoun
and Smart, 2005; Fry, 2008). P. infestans is a hemibiotrophic
pathogen that initially requires living host cells but then causes
extensive necrosis of host tissue culminating in profuse sporo-
lation (Kamoun and Smart, 2005). During the biotrophic phase,
the pathogen establishes intimate associations with host cells
through the production of digit-like haustoria, structures that
function in host translocation of effector proteins and probably
nutrient uptake (Birch et al., 2006; Whisson et al., 2007).

Like other oomycetes, P. infestans is predicted to secrete
hundreds of effector proteins that target two distinct sites in the
host plant (Kamoun, 2006; Whisson et al., 2007; Haas et al.,
2009). Apoplastic effectors are secreted into the plant extracel-
larular space, whereas cytoplasmic effectors are translocated into
the plant cell, where they target different subcellular compart-
ments. In contrast with apoplastic effectors, which are known to
inhibit host hydrolases (Tian et al., 2004, 2005, 2007; Damasceno
et al., 2008), the biochemical activities of cytoplasmic effectors
remain poorly understood. Oomycete cytoplasmic effectors are
modular proteins that carry N-terminal signal peptides followed
by conserved motifs, notably the RXLR and LXLFLAK motifs
(Birch et al., 2006; Kamoun, 2006; Tyler et al., 2006; Kamoun,
2007; Morgan and Kamoun, 2007; Win et al., 2007; Birch et al.,
2008). The RXLR motif defines a domain, similar to a host
translocation signal of malaria parasites, that enables delivery of
effector proteins inside plant cells (Bhattacharjee et al., 2006;
Whisson et al., 2007; Dou et al., 2008b; Grouffaud et al., 2008).
One of the best-studied oomycete RXLR effectors is P. infestans
AVR3a, which confers avirulence on potato plants carrying the
R3a gene (Armstrong et al., 2005). In addition to its avirulence
activity, AVR3a suppresses the cell death induced by INF1
elicitin, another secreted protein of P. infestans with features of
pathogen-associated molecular patterns (PAMPs) (Bos et al.,
2006, 2009). AVR3a is thought to contribute to virulence through
this PAMP suppression activity (Bos et al., 2009).

More than a dozen late blight resistance genes (R genes) have
been introgressed into cultivated potato from wild species such as
Solanum demissum, Solanum bulbocastanum, and Solanum
berthaultii using classical breeding (Fry, 2008). Some of these R
genes, notably S. demissum R1 and R3a as well as S. bulboco-
astanum Rpi-blb1 (also known as RB) and Rpi-blb2, have been
cloned (Ballvora et al., 2002; Song et al., 2003; van der Vossen
et al., 2003, 2005; Huang et al., 2005; Kuang et al., 2005;
Vleeshouwers et al., 2008; Wang et al., 2008). Although late blight
R genes have long been noted to be ineffective in the field over
long periods of time, empirical observations backed by plausible
hypotheses indicate that some of the newly cloned R genes
could mediate resistance in a durable enough fashion to prove
useful in agriculture (Helgeson et al., 1998; Song et al., 2003;
van der Vossen et al., 2003). For example, Rpi-blb1 recognizes
a broad spectrum of P. infestans isolates and has proven
effective in the field in several geographical areas and over
several growing seasons (Helgeson et al., 1998; Song et al.,
2003; van der Vossen et al., 2003; Kuhl et al., 2007; Halterman
et al., 2008). This has prompted interest in the deployment of
potato cultivars with these novel R genes. A transgenic potato
variety carrying Rpi-blb1 and Rpi-blb2 has entered the commer-
cialization pipeline in Europe (Vleeshouwers et al., 2008), and
other initiatives to release these genes in several developing
countries are under way (USAID Agricultural Biotechnology
Support Project II, http://www.absp2.cornell.edu). The identifi-
cation of the Avr genes targeted by these R genes would help to
determine the extent to which broad-spectrum resistance differs
from other types of resistance and will generate the tools to
monitor P. infestans populations for mutations in the Avr genes
(Kamoun and Smart, 2005; Vleeshouwers et al., 2008).

The discovery that oomycete AVR proteins belong to the RXLR
effector class creates the opportunity to use bioinformatics to
predict a robust set of candidate effectors. In this study, we
combined allele mining with high-throughput in planta expres-
sion to assess the activities of 62 RXLR effector homologs from
P. infestans. This effectoromics approach revealed that 16 of the
62 effectors cause phenotypes when expressed in planta. Four
distinct effector activities were observed: (1) suppression of INF1
triggered cell death, (2) nonspecific induction of weak cell death
response in Nicotiana benthamiana and other solaceous
plants, (3) specific induction of HR cell death in the presence of
Rpi-blb1 and (4) specific induction of HR cell death in the
presence of Rpi-blb2. The latter two activities are expected for
Avrblb1 and Avrblb2. The AVRblb2 family was then studied in
more detail revealing that a single amino acid site under positive
selection in P. infestans is critical for recognition by Rpi-blb2.
A subset of the infection-ready library we describe here was
previously used to screen a collection of Solanum genotypes
for induction of HR-like symptoms and resulted in the indepen-
dent discovery of Avrblb1 (Vleeshouwers et al., 2008).

RESULTS

Strategy for Allele Mining and in Planta Expression of
P. infestans RXLR Effectors

To identify RXLR effectors with novel activities, we devised a
strategy that combines allele mining with in planta expression
(Figure 1). In brief, primer pairs based on the mature region of
candidate RXLR effectors (without the signal peptide) were
previously used to screen a collection of
Solanum demissum, Solanum bulbocastanum, and Solanum
berthaultii using classical breeding (Fry, 2008). Some of these R
genes, notably S. demissum R1 and R3a as well as S. bulboco-
astanum Rpi-blb1 (also known as RB) and Rpi-blb2, have been
cloned (Ballvora et al., 2002; Song et al., 2003; van der Vossen
et al., 2003, 2005; Huang et al., 2005; Kuang et al., 2005;
Vleeshouwers et al., 2008; Wang et al., 2008). Although late blight
R genes have long been noted to be ineffective in the field over
long periods of time, empirical observations backed by plausible
hypotheses indicate that some of the newly cloned R genes
could mediate resistance in a durable enough fashion to prove
useful in agriculture (Helgeson et al., 1998; Song et al., 2003;
van der Vossen et al., 2003). For example, Rpi-blb1 recognizes
a broad spectrum of P. infestans isolates and has proven
effective in the field in several geographical areas and over
several growing seasons (Helgeson et al., 1998; Song et al.,
The genes, named following cloning in the PVX vector pGR106 (Table 1; full description in Supplemental Data Set 1 online). We determined that 53 of the 62 sequences could be grouped in 15 families with 2 to 21 sequences per family (see Supplemental Table 3 online). Because closely related sequences could correspond to either alleles or paralogs, we will refer to them as homologs.

Over Half the Examined RXLR Effector Genes Are Polymorphic

Of the 32 PexRD genes examined, 18 (56%) turned out to be polymorphic among the examined P. infestans isolates (Table 1). Of these, 13 genes displayed nonsynonymous amino acid polymorphisms, four had premature stop codons when compared with the parental EST, whereas one gene had only silent mutations (synonymous amino acid substitutions). These results are consistent with the rapid evolutionary rates associated with RXLR effectors (Tyler et al., 2006; Win et al., 2007) and also indicate that the majority of the observed polymorphisms are expected to be functionally relevant. As reported earlier in a genome-wide analysis of RXLR effector paralogs of Phytophthora sojae, Phytophthora ramorum, and Hyaloperonospora arabidopsis (Win et al., 2007), most of the polymorphisms localized to the C-terminal region of the effectors, and the RXLR and EER motifs were invariably conserved across the homologs (Table 1; see Supplemental Data Set 1 online).

The Majority of the Selected RXLR Effector Genes Are Expressed during Infection of Tomato

To determine the extent to which the P. infestans PexRD genes are expressed during colonization of plants, we analyzed the expression of the 32 genes during the interaction of P. infestans with its host plant tomato using RT-PCR analyses (see Supplemental Figure 1 online). Total RNA was isolated from leaves of tomato 0, 1, 2, 3, 4, and 5 d after inoculation (DAI) with two different P. infestans isolates, 90128 and 88069, and from P. infestans mycelium grown in vitro. The constitutive elongation factor 2 alpha (ef2α) (Torto et al., 2002) and the in planta–induced Kazal-like protease inhibitor gene ep11 (Tian et al., 2004) were used as controls. We detected transcripts for 30 of the 32 genes in at least one of the examined stages (see Supplemental Figure 1 online). Among these, 29 genes were expressed during colonization of tomato, whereas transcripts for PexRD4 were detected only in mycelium (see Supplemental Figure 1 online). Transcripts for nine genes, PexRD3, PexRD6/ipIO, PexRD8, PexRD24, PexRD31, PexRD44, PexRD45, PexRD49, and PexRD50, were detected in the infection time points but not in mycelium (see Supplemental Figure 1 online; summarized in Table 1). These results show that the great majority of the selected RXLR effector candidate genes are expressed during infection of tomato, consistent with their predicted function.

In addition, we cross-checked our gene list with the RXLR effector genes previously reported to be induced during infection of potato using real-time PCR (Whisson et al., 2007) or using Nimblegen oligonucleotide microarrays (Haas et al., 2009). Of the 32 PexRD genes, 22 were shown by Whisson et al. (2007) and 16 by Haas et al. (2009) to be induced during infection of potato (see Supplemental Table 4 online). These expression data
independently confirm the in planta (tomato and potato) expression pattern for 27 out of the 32 candidate RXLR effector genes.

### Functional Validation of the Signal Peptides of RXLR Effectors

To validate functionally the signal peptide predictions of the selected RXLR effector genes, we used a genetic assay based on the requirement of yeast cells for invertase secretion to grow on sucrose or raffinose media (Klein et al., 1996; Jacobs et al., 1997; Lee et al., 2006). The predicted signal peptide sequences and the subsequent two amino acids of four PEXRD genes, PexRD6, ipiO, AvrBlb1, PexRD7, Avr3a, Pex147-2, Avr3a paralog, and Pex147-3, Avr3a paralog, were fused in frame to the mature sequence of yeast invertase in the vector pSUC2 (Jacobs et al., 1997) (see Supplemental Table 5 online). All four PexRD constructs enabled the invertase mutant yeast strain YTK12 to grow on YPRAA medium (with raffinose instead of sucrose, growth only when invertase is secreted) (Figure 2). In addition, invertase secretion was confirmed with an enzymatic activity test based on reduction of the dye 2,3,5-triphenyltetrazolium chloride (TTC) to the insoluble red colored triphenylformazan (Figure 2). By contrast, the negative control yeast strains did not grow on YPRAA, and the TTC-treated culture filtrates remained colorless (Figure 2). These results indicate that the signal peptides of PexRD6, PexRD8, PexRD39, and PexRD40 are functional and confirm earlier observations that predictions obtained with the SignalP program are highly accurate (Menne et al., 2000; Schneider and Fechner, 2004; Lee et al., 2006).

### PexRD8 and PexRD3645-1 Suppress the Hypersensitive Cell Death Induced by INF1

Suppression of plant innate immunity, particularly PAMP-triggered immunity, has emerged as a common function of phytopathogen effectors (Block et al., 2008; Hogenhout et al., 2009). Elicitins are structurally conserved proteins in oomycetes that trigger defenses in a variety of solanaceous plants and have features of PAMPs (Nurnberger and Brunner, 2002; Vleeshouwers et al., 2006). Previously, we showed that the P. infestans

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### Table 1. Description of the Selected PexRD Genes

<table>
<thead>
<tr>
<th>Gene Name(s)</th>
<th>Number of Homologs Amplified</th>
<th>Type of Mutations</th>
<th>SignalP HMM Probabilitya</th>
<th>SignalP NN Mean S Scorea</th>
<th>SignalP Lengtha RXLR dEER</th>
<th>Expression in Vitro (Mycelium)</th>
<th>Expression in Tomato (Infection)</th>
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<tr>
<td>PexRD1</td>
<td>1</td>
<td>None detected</td>
<td>0.989</td>
<td>0.654</td>
<td>19 RQLR EDGEER +</td>
<td>+</td>
<td></td>
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<tr>
<td>PexRD2</td>
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<td>None detected</td>
<td>0.998</td>
<td>0.913</td>
<td>20 RLLR ENDDDSEAR +</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>PexRD3</td>
<td>1</td>
<td>None detected</td>
<td>0.998</td>
<td>0.741</td>
<td>23 RFLR EGDNIEER –</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>PexRD4</td>
<td>1</td>
<td>None detected</td>
<td>0.998</td>
<td>0.813</td>
<td>21 RFLR DEER –</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>PexRD6, ipiO, AvrBlb1</td>
<td>3</td>
<td>Nonsynonymous</td>
<td>1.000</td>
<td>0.968</td>
<td>21 RSLR DEER –</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>PexRD7, Avr3a</td>
<td>2</td>
<td>Nonsynonymous</td>
<td>0.998</td>
<td>0.745</td>
<td>21 RLLR EENEETSEER +</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Pex147-2, Avr3a paralog</td>
<td>1</td>
<td>None detected</td>
<td>0.991</td>
<td>0.725</td>
<td>21 RLLR ESEEETSEER –</td>
<td>–</td>
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</tr>
<tr>
<td>Pex147-3, Avr3a paralog</td>
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<td>None detected</td>
<td>0.992</td>
<td>0.742</td>
<td>21 RFLR EENEETSEER –</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>PexRD8</td>
<td>1</td>
<td>None detected</td>
<td>0.989</td>
<td>0.832</td>
<td>22 RLLR DDDDEEER –</td>
<td>+</td>
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</tr>
<tr>
<td>PexRD10</td>
<td>1</td>
<td>None detected</td>
<td>0.998</td>
<td>0.925</td>
<td>19 RKL R EER +</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>PexRD11</td>
<td>2</td>
<td>Premature stop</td>
<td>1.000</td>
<td>0.907</td>
<td>21 RLLR DEGELTEER +</td>
<td>+</td>
<td></td>
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<tr>
<td>PexRD12</td>
<td>2</td>
<td>Synonymous</td>
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<td>0.869</td>
<td>22 RSLR DSDDGEER +</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>PexRD13</td>
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<td>Premature stop</td>
<td>1.000</td>
<td>0.843</td>
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<td>+</td>
<td></td>
</tr>
<tr>
<td>PexRD14</td>
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<td>0.781</td>
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<td>+</td>
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<tr>
<td>PexRD16</td>
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<td>Nonsynonymous</td>
<td>1.000</td>
<td>0.951</td>
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<td>+</td>
<td></td>
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<tr>
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<td>0.960</td>
<td>0.525</td>
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<td>+</td>
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</tr>
<tr>
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<td>None detected</td>
<td>0.993</td>
<td>0.921</td>
<td>21 RLLR EREVEGEE +</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>PexRD22</td>
<td>2</td>
<td>Nonsynonymous</td>
<td>0.988</td>
<td>0.916</td>
<td>17 RFLR EDASDEER +</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>PexRD24</td>
<td>2</td>
<td>Nonsynonymous</td>
<td>1.000</td>
<td>0.901</td>
<td>22 RSLR ETESEDEER –</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>PexRD26</td>
<td>2</td>
<td>Nonsynonymous</td>
<td>0.981</td>
<td>0.890</td>
<td>22 RVL R DEER +</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>PexRD27</td>
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<td>None detected</td>
<td>0.992</td>
<td>0.885</td>
<td>28 RLR DSEEER –</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>PexRD28</td>
<td>1</td>
<td>None detected</td>
<td>0.999</td>
<td>0.916</td>
<td>24 RSLR ETESEDEER –</td>
<td>–</td>
<td></td>
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<tr>
<td>PexRD31</td>
<td>1</td>
<td>None detected</td>
<td>0.986</td>
<td>0.672</td>
<td>28 RSLR EDEQDEER –</td>
<td>–</td>
<td></td>
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<tr>
<td>PexRD36</td>
<td>2</td>
<td>Premature stop</td>
<td>0.999</td>
<td>0.881</td>
<td>22 RHLR DDEER +</td>
<td>+</td>
<td></td>
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<tr>
<td>PexRD39, AvrBlb2</td>
<td>13b</td>
<td>Nonsynonymous</td>
<td>1.000</td>
<td>0.864</td>
<td>22 RSLR –</td>
<td>+</td>
<td></td>
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<tr>
<td>PexRD40, AvrBlb2</td>
<td>13b</td>
<td>Nonsynonymous</td>
<td>1.000</td>
<td>0.857</td>
<td>22 RSLR –</td>
<td>+</td>
<td></td>
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<tr>
<td>PexRD41</td>
<td>3</td>
<td>Nonsynonymous</td>
<td>1.000</td>
<td>0.849</td>
<td>21 RSLR –</td>
<td>+</td>
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</tr>
<tr>
<td>PexRD44</td>
<td>1</td>
<td>None detected</td>
<td>1.000</td>
<td>0.949</td>
<td>21 RFLR QEEGVFEER –</td>
<td>+</td>
<td></td>
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<tr>
<td>PexRD45</td>
<td>2</td>
<td>Premature stop</td>
<td>0.999</td>
<td>0.782</td>
<td>22 RSLR –</td>
<td>–</td>
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<tr>
<td>PexRD46</td>
<td>3</td>
<td>Nonsynonymous</td>
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<td>0.854</td>
<td>21 RSLR –</td>
<td>+</td>
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<td>PexRD49</td>
<td>1</td>
<td>None detected</td>
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<td>20 RLLR EER –</td>
<td>–</td>
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<tr>
<td>PexRD50</td>
<td>2</td>
<td>Nonsynonymous</td>
<td>1.000</td>
<td>0.875</td>
<td>20 RLLR –</td>
<td>–</td>
<td></td>
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</tbody>
</table>

aS-mean value, HMM score, and signal peptide length predicted using SignalPv2.0 (http://www.cbs.dtu.dk/services/SignalP-2.0).
bPrimers for both PexRD39 and PexRD40 amplified the same homologs.
RXLR effector AVR3a suppresses the cell death induced by INF1 elicitation in *N. benthamiana* (Bos et al., 2006, 2009). To identify other RXLR effectors that suppress INF1 cell death, we infiltrated *A. tumefaciens* strains carrying the 62 pGR106-PexRD constructs and the negative control pGR106-GFP (for green fluorescent protein) in *N. benthamiana* leaves to express the candidate suppressors. One day later, the infiltration sites were challenged with an *A. tumefaciens* strain carrying the p35S-INF1 construct, and cell death symptoms were scored 3 to 5 d later. Phenotypic evaluation of the infiltrated sites revealed that two clones, pGR106-PexRD8 and pGR106-PexRD3645-1, reduced the rate of INF1 cell death to below 50% compared with >90% for the control pGR106-GFP and <15% for pGR106- AVR3aKI (see Supplemental Figure 2 online).

To validate the results of the screen, we performed additional side-by-side assays to compare the suppression activities of PexRD8 and PexRD3645-1 to that of AVR3aKI (Figure 3). These results confirmed that PexRD8 and PexRD3645-1 consistently suppress the HR induced by INF1, although not to the level achieved by AVR3aKI. We conclude that PexRD8 and PexRD3645-1 carry INF1 cell death suppression activity.

We also screened our pGR106-PexRD library for suppression of the necrosis induced by the *P. infestans* Nep1-like protein NPP1.1, a protein that appears to function as a toxin during the necrotrophic phase of the infection (Kanneganti et al., 2006; Qutob et al., 2006). None of the 62 clones reproducibly suppressed NPP1.1-mediated necrosis (data not shown).

**PexRD2 Induces a Weak Cell Death Response in *N. benthamiana***

Ectopic expression of effector genes in plant cells often leads to macroscopic phenotypes such as cell death, chlorosis, and tissue browning when expressed in host cells (Kjemtrup et al., 2000; Torto et al., 2003; Cunnac et al., 2009; Gurlebeck et al., 2009; Haas et al., 2009). To identify PexRD genes that induce phenotypic symptoms in plants, we individually inoculated the *A. tumefaciens* strains carrying the 62 pGR106-PexRD plasmids on *N. benthamiana* using both the wounding (toothpick) and agro-infiltration assays (Huitema et al., 2004; Bos et al., 2009). Only pGR106-PexRD2 induced a weak delayed necrotic response appearing at 7 to 10 DAI in the toothpick assay (Figure 4A).
addition, the necrotic area was reduced relative to the HR induced by the positive control pGR106-INF1 (Figure 4A).

To determine whether enhanced expression of PexRD2 results in enhanced cell death inducing activity, we coexpressed the pGR106-PexRD2 construct with a construct expressing p19, a suppressor of posttranscriptional gene silencing from Tomato bushy stunt virus that is known to increase gene expression in the agroinfiltration assay (Voinnet et al., 2003). We observed that 3 to 5 d after infiltration, the PexRD2-associated cell death was accelerated and enhanced in the presence of p19 (Figure 4B). We conclude that the cell death induced by PexRD2 is probably dose dependent.

The ubiquitin ligase-associated protein SGT1 is required for a variety of cell death responses in plants (Austin et al., 2002; Azavedo et al., 2002; Peart et al., 2002; Kanneganti et al., 2006). We tested whether SGT1 is required for PexRD2-induced cell death using virus-induced gene silencing (VIGS) with Tobacco rattle virus (TRV) followed by agroinfiltration assays (Hultema et al., 2004). SGT1-silenced and control plants were infiltrated with A. tumefaciens strains containing pGR106-PexRD2 mixed with (+) p19 or without (−) p19 (Figures 4C and 4D). Silencing of SGT1 suppressed the cell death response induced by PexRD2, indicating that similar to a variety of other effectors, PexRD2 requires SGT1 to elicit cell death in N. benthamiana.

**Functional Identification of Avrblb1 and Avrblb2**

We next used the PVX-based high-throughput assay to identify the Avr genes matching the S. bulbocastanum R genes Rpi-blb1 and Rpi-blb2 (van der Vossen et al., 2003, 2005). First, we infiltrated leaves of N. benthamiana with A. tumefaciens strains carrying one of the two R genes. Two days later, the leaves were wound inoculated in triplicate with each of the 62 pGR106-PexRD A. tumefaciens strains. The hypersensitive cell death

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**Figure 3.** PexRD8 and PexRD3645-1 Suppress the HR Induced by P. infestans INF1 Elicitin.

(A) and (B) Agroinfiltration sites in N. benthamiana leaves expressing either PexRD8 (A) or PexRD3645-1 (B) were challenged with A. tumefaciens expressing the INF1 elicitor. The INF1-induced cell death was scored at 3 and 4 DAI. Two independent pGR106-derived clones of PexRD8 and PexRD3645-1 were used (bottom panels; clone #1 on the bottom left side and #2 on the bottom right). A. tumefaciens strain carrying pGR106-dGFP (dGFP) was used as a negative control, and pGR106-avr3a (AVR3a) was used as a positive control.

(C) and (D) Quantification of suppression of INF1 cell death by PexRD8 and PexRD3645-1 relative to AVR3a. The mean percentages of sites showing cell death and the standard errors were scored from 20 infiltration sites based on three independent experiments using N. benthamiana leaves expressing either PexRD8 (C) or PexRD3645-1 (D). Two independent pGR106-derived clones of PexRD8 and PexRD3645-1 were used (#1 and #2) as shown in (A) and (B).
responses were monitored up to 14 DAI. The screens revealed that two PexRD6/IpiO clones triggered HR-like lesions on Rpi-blb1 expressing leaves, and 10 clones of the closely related PexRD39 and PexRD40 clones triggered HR on Rpi-blb2 leaves (Figure 5A; see Supplemental Data Set 1 online).

To confirm these results using a different assay, we performed coagroinfiltration of the two PexRD6/IpiO and two of the PexRD39/40 A. tumefaciens pGR106 strains with the two R genes strains in N. benthamiana. The HR reactions observed in the wound inoculation screen were confirmed (Figures 5B to 5D). In the Rpi-blb1 coinfiltrations, the HR was observed with the two PexRD6/IpiO clones starting at 4 DAI, and for Rpi-blb2, the HR was observed with both PexRD39 and PexRD40 constructs starting at 3 DAI (Figures 5B to 5D). Altogether, these experiments indicate that the identified clones are specifically recognized by the cognate R genes. We suggest that PexRD6/IpiO is Avrblb1 and PexRD39/40 is Avrblb2.

The PexRD6/IpiO gene was independently identified as Avrblb1 by Vleeshouwers et al. (2008) using a functional screen on wild Solanum plants carrying the Rpi-blb1 gene. In both studies, PexRD641-3 (named IpiO1-K143N by Vleeshouwers et al., 2008) and PexRD641-10 (IpiO2) caused the HR on Rpi-blb1-expressing leaves, whereas homolog PexRD639-6 (IpiO4) failed to trigger cell death (see Supplemental Data Set 1 online).

The PexRD39 and PexRD40 genes are close homologs with open reading frames of 303 bp, corresponding to predicted translated products of 100 amino acids. The two predicted proteins differ only in 9 out of 100 amino acids, seven of which are in the mature proteins. Primers based on these two genes amplified overlapping sets of amplicons corresponding to 13 different sequences (see Supplemental Data Set 1 online). Of these, 10 different clones induced the HR on Rpi-blb2-expressing leaves in both wounding and agroinfiltration assays, whereas PexRD3989-2, PexRD3989-7, and PexRD39159-6 did not (see Supplemental Data Set 1 online).

PexRD39 and PexRD40 are also similar to other RXLR effectors, namely, PexRD41, PexRD45, and PexRD46 (BLASTP E values < 1e-05), resulting in a superfamily of 21 proteins (see Figure 4. PexRD2 Promotes Cell Death in N. benthamiana.

(A) Symptoms observed in N. benthamiana after wound inoculation with A. tumefaciens carrying pGR106 vector derivatives expressing a subset of the 62 RXLR effectors of P. infestans. The negative and positive controls were A. tumefaciens strains carrying pGR106-dGFP (dGFP) and pGR106-INF1, respectively. Note the small ring of dead cells triggered by the pGR106-PexRD2 strain relative to the more expanded cell death triggered by pGR106-INF1. All strains were inoculated in triplicate. The photo was taken 12 DAI.

(B) The PexRD2-associated cell death is enhanced in the presence of gene silencing suppressor p19. A. tumefaciens carrying pGR106-

PexRD2 was mixed with (+) p19 or without (−) an A. tumefaciens p19 strain and infiltrated into N. benthamiana leaves. The experiment was repeated three times with similar results. After 6 d, the PexRD2-associated cell death symptoms were observed in both cases but were enhanced in the presence of p19. All strains were inoculated in triplicate.

(C) SGT1 is required for the cell death response induced by PexRD2. Leaves of N. benthamiana vector control (TRV2-dGFP) and SGT1-silenced (TRV2-NbSGT1) plants were challenged by agroinfiltration of A. tumefaciens carrying pGR106-dGFP (dGFP, negative control) or pGR106-PexRD2. Control-silenced plants showed symptoms of the cell death induced by the PexRD2 starting at 3 to 5 DAI, and this response was enhanced in the presence of gene silencing suppressor p19 (left panel). In the TRV2-NbSGT1 plants, the PexRD2-associated cell death was suppressed (right panel).

(D) RT-PCR analysis of SGT1 expression in control (TRV2-dGFP) and SGT1-silenced (TRV2-NbSGT1) N. benthamiana. Total RNA was extracted from the silenced plants and subjected to RT-PCR analysis with SGT1 primers to detect SGT1 transcripts. The Actin gene was used to confirm equal total RNA amounts among samples. Similar results were obtained at least two times independent experiments.
Supplemental Table 3 online). However, none of these additional homologs induced the HR on Rpi-blb2-expressing leaves.

**The Avrblb2 Family Is Highly Variable and under Diversifying Selection in P. infestans**

We elected to study the Avrblb2 family in more detail because the forthcoming release of potato cultivars carrying Rpi-blb2 would benefit from a better understanding of the targeted effector. To mine further sequence polymorphisms of Avrblb2 in P. infestans, we used the strategy that we previously applied for the small Cys-rich protein SCR74 (Liu et al., 2005). We performed PCR amplifications with genomic DNA from six diverse P. infestans isolates, 88069, 90128, IPO-0, IPO-428, IPO-566, and US980008 (Table 2; see Supplemental Table 2 online). Direct sequencing of amplicons obtained from genomic DNA of the six isolates resulted in mixed sequences, indicating that the primers amplified multiple alleles or paralogs of Avrblb2. Therefore, we cloned the amplicons and generated high-quality sequences (phred Q>20, phred software; CodonCode) of the inserts of 85 different clones. In addition, we included seven Avrblb2 paralogous sequences from the genome sequence of strain P. infestans T30-4 (Haas et al., 2009).

A total of 24 different nucleotide sequences, encoding 19 predicted amino acid sequences, could be identified for Avrblb2 (Figure 6A, Table 2; see Supplemental Data Set 2 online). Polymorphisms were detected in 24 of the 279 examined nucleotides. None of the Avrblb2 sequences contained premature stop codons or frameshift mutations. Multiple alignments of the 24 predicted AVRblb2 amino acid sequences revealed a highly polymorphic family (Figure 6A). A total of 14 polymorphic amino acid sites were identified, 10 of which localize to the C-terminal domain (after the RSLR motif).

To determine the selection pressures underlying sequence diversification in the AVRblb2 family, we calculated the rates of non-synonymous ($d_N$) and synonymous ($d_S$) mutations across the 24 sequences. We found that $d_N$ was greater than $d_S$ ($v = d_N/d_S > 1$) in 121 of 276 pairwise comparisons (see Supplemental Figure 3 and Supplemental Data Set 3 online). In the C-terminal (after RSLR) domain (after the RSLR)

**Figure 5.** Functional Identification of Avrblb1 and Avrblb2.

(A) Wound inoculation screening of the pGR106-PexRD library on N. benthamiana leaves expressing the S. bulbocastanum R genes Rpi-blb1 (left panel) and Rpi-blb2 (right panel). The two HR-inducing PexRD6/IpiO clones (PexRD641.3/IpiO1-K143N and PexRD641.10/IpiO2) and two of the positive PexRD39 and PexRD40 clones (PexRD39101-4, and PexRD4015-3) are shown. Additional PexRD clones that yielded negative responses are also shown. All tested clones are labeled RD# for the corresponding PexRD clone number. The negative and positive controls were A. tumefaciens strains carrying pGR106-ΔGFP (ΔGFP) and pGR106-PINP1 (NPP1), respectively.

(B) to (D) Confirmation of Avrblb cloning using agroinfiltration. Agroinfiltration of the positive A. tumefaciens strains carrying Avrblb1 (PexRD641.3/IpiO1-K143N and PexRD641.10/IpiO2, top and bottom right panels, respectively) and Avrblb2 (PexRD39 and PexRD40, top and bottom panels, respectively) was performed in N. benthamiana corresponding to control plants (B) or leaves expressing Rpi-blb1 (C) or Avrblb2 (D). A. tumefaciens strain carrying pGR106-ΔGFP (ΔGFP) was used as a negative control (top and bottom left panels of leaves). Coinfiltration was performed with A. tumefaciens solutions mixed in 1:2 ratio (Avr:R gene). Hypersensitive cell death was observed starting at 4 DAI, and the photograph was taken at 7 DAI. The experiment was repeated three times with similar results.
protein regions, \(d_N \) exceeded \(d_S \) in 71 over 276 possible pairwise comparisons (162 bp) (see Supplemental Figure 3 online). These results provide evidence that positive diversifying selection has acted on the AVRblb2 family, particularly on the C-terminal effector domain.

AVRblb2 Residues under Diversifying Selection

To detect the particular amino acid sites under diversifying selection in the AVRblb2 family, we applied the maximum likelihood (ML) method implemented in the PAML 4.2a software package (Nielsen and Yang, 1998; Yang et al., 2000; Yang, 2007). The discrete model M3 with three site classes revealed that 12% of the amino acid sites were under strong positive selection with \(\nu_2 = 12.32\). The likelihood ratio test (LRT) for comparing M3 with M0 is 2 \(D_L = 2 \times [-607.52 - (-622.81)] = 45.74\), which is greater than the \(\chi^2\) critical value (9.21 at 1% significance level, with degrees of freedom = 4) (Table 3). This indicates that the discrete model M3 fits the data significantly better than the neutral model M0, which does not allow for the presence of diversifying selection sites with \(\nu > 1\). We then used the empirical Bayes theorem to identify eight amino acid sites (40V, 42P, 47I, 69A, 70Q, 84G, 88E, and 95A) implicated as being under diversifying selection with >95% confidence under the discrete model M3 (Table 3).

We also proceeded to analyze paralogous sequences following the strategy of Win et al. (2007). Using the same ML methods described above, we analyzed a subset of four paralog sequences of \(P. \infestans\) T30-4 and, remarkably, identified only a single position, amino acid 69, under positive selection (Figure 6C). This indicates that residue 69 can be detected as a positively selected amino acid even using less sensitive analyses and a smaller set of sequences.

AVRblb2 Does Not Require the RXLR Motif for Perception by Rpi-blb2

RXLR effectors are modular proteins with the effector activity carried by the C-terminal domain that follows the RXLR region.

Table 2. Distribution of Avrblb2 Sequences among \(P. \infestans\) Isolates

<table>
<thead>
<tr>
<th>Homolog ID</th>
<th>Amino Acid at Position 69</th>
<th>(P. \infestans) Isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>T30-4(^a)</td>
<td>PITG 04090 CV89</td>
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<tr>
<td>D5</td>
<td>Ala</td>
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<td>F2</td>
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<tr>
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<tr>
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<td></td>
</tr>
<tr>
<td>X12</td>
<td>Phe</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\)The descriptors in this column correspond to the gene ID of the Avrblb2 paralogs present in the reference strain T30-4 (Haas et al., 2009).

\(^b\)The descriptors in these columns correspond to the clone IDs recovered from each of the strains for each one of the 24 Avrblb2 homologs.

\(\nu_2\) is the parameter of the \(\beta\)-distribution, and \(\nu = \nu_1 + \nu_2\) is the parameter of the \(\beta+\nu\)-distribution.
The RXLR motif is not required for avirulence activity when the protein is directly expressed inside plant cells (Bos et al., 2006; Allen et al., 2008). However, Dou et al. (2008a) showed that the RXLR motif of *P. sojae* Avr1b is required for cell death induction when a full-length construct with the signal peptide is expressed in plant cells, presumably to enable reentry of the protein following secretion. We cloned a full-length AvrBlb2 (PexRD40170-7), with its native signal peptide, in the binary PVX vector and found by agroinfiltration that it triggers Rpi-blb2–dependent HR in *N. benthamiana* (Figure 7).

Figure 6. The AVRBlb2 Family Is Highly Polymorphic and under Diversifying Selection in *P. infestans*.

(A) Multiple sequence alignment of 24 AVRBlb2 amino acid sequences from *P. infestans*. Single-letter amino acid codes were used. Residue numbers are denoted above the sequences. The predicted signal peptide, RXLR motif, and 34–amino acid functional domains are indicated above the alignment.

(B) Posterior probabilities along the AVRBlb2 protein sequence for site classes estimated under the discrete model M8 in the PAML software. The analysis was based on the 24 identified AVRBlb2 sequences described in Figure 6A. Amino acid sites 42P, 47I, 69A, 70Q, 84G, 88E, and 95A marked in red have high posterior probabilities (P > 0.95 and \( \omega > 8.9 \)) and are potentially under positive selection.

(C) Posterior probabilities along the AVRBlb2 protein sequence obtained with a subset of four paralogous sequences from *P. infestans* T30-4 strain. In this analysis, only residue 69A (\( \omega = 69.434 \)) is under positive selection. The position of the signal peptide, RXLR motif, and the 34–amino acid domain are indicated below the graphs.
mutated this sequence into ASAA. Agroinfiltration of the mutated Avrblb2 with Rpi-bib2 in N. benthamiana resulted in a confluent HR similar to the response triggered by the wild-type Avrblb2 (Figure 7). To account for the possibility that the native AVRblb2 signal peptide is not fully effective in plants and to avoid potential problems due to the PVX expression system, we made new constructs in the A. tumefaciens binary vector pCB302-3. The two constructs (RSLR and ASAA mutants), consisting of a fusion between the signal peptide of the tomato Ser protease P69B (Tian et al., 2004) and the mature portion of PexRD40. The corresponding pGR106-PexRD constructs were used in agroinfiltrations of N. benthamiana (referred to as PexRD40 from here on), from Val to Ala, Ile, or Phe and constructed a fusion between the FLAG epitope tag and amino acid C-terminal region of AVRblb2 excludes the RXLR leader sequence but, interestingly, includes the one polymorphic amino acid at position 69(V) that was identified as positively selected in the ML method (Figure 6).

### Deletion Analysis of AVRblb2 Identifies a 34–Amino Acid Region Sufficient for Induction of Rpi-bib2–Mediated Cell Death

To delineate the functional domain of AVRblb2, we made a series of deletion constructs and assayed them in N. benthamiana (Figure 7). Results obtained with our original pGR106-PexRD constructs indicate that the AVRblb2 homologs do not require a signal peptide sequence to trigger Rpi-bib2–mediated HR (Figure 5) and that the recognition event occurs inside the plant cytoplasm similar to the AVR3a and R3a interaction (Armstrong et al., 2005; Bos et al., 2006). We assayed five N-terminal and C-terminal deletion mutants for activation of Rpi-bib2 cell death by agroinfiltration in N. benthamiana. These experiments indicated that a 34–amino acid C-terminal region of AVRblb2 (EAQEVIQSGRGDGYGGFWKNVVQSTNKIVKKPDI) is sufficient for triggering Rpi-bib2–mediated cell death (Figure 7). This 34–amino acid C-terminal region of AVRblb2 excludes the RXLR leader sequence but, interestingly, includes the one polymorphic amino acid at position 69(V) that was identified as positively selected in the ML method (Figure 6).

### The Positively Selected Amino Acid 69 of AVRblb2 Is Critical for Activation of Rpi-bib2 Hypersensitivity

The positively selected residue 69 is the only polymorphic residue within the 34–amino acid region that correlates with the HR-inducing activity on Rpi-bib2–expressing leaves. The 10 AVRblb2 homologs that are recognized by Rpi-bib2 have Val-69, Ala-69, or Ile-69, whereas the three that are not recognized have Phe-69. To further evaluate the impact of residue 69 on AVRblb2 activity, we mutated this residue in PexRD40170-7 (referred to as PexRD40 from here on), from Val to Ala, Ile, or Phe and constructed a fusion between the FLAG epitope tag and the mature portion of PexRD40. The corresponding pGR106-FLAG–PexRD40 constructs were used in agroinfiltrations of N. benthamiana to express the mature PexRD40 proteins (amino acids 23 to 100) in combination with Rpi-bib2 (Figure 8). In contrast with PexRD40, PexRD40V69A, and PexRD40V69I, the PexRD40V69F mutant consistently failed to induce Rpi-bib2–mediated hypersensitivity in side-by-side infiltrations (Figures 8B to 8D). Protein gel blot hybridizations of extracts from infiltrated

### Table 3. Likelihood Ratio Test Results for Avrblb2

<table>
<thead>
<tr>
<th>Model</th>
<th>Estimate Parameters</th>
<th>InL</th>
<th>Sites under Selection</th>
<th>Degree of Freedom</th>
<th>Model Comparison</th>
<th>2ΔL</th>
<th>χ² Critical Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>M0: one ratio</td>
<td>P0 = 0.82144 P1 = 0.05225</td>
<td>630.39</td>
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<td>M3: discrete</td>
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<tr>
<td>M7: β</td>
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<td>M7 vs. M8</td>
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<tr>
<td>M8: β + w</td>
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<td>42P 47I 69A 70Q 84G 88E 95A</td>
<td></td>
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a InL, log likelihood value.
b Amino acid sites inferred to be under positive selection with a probability >99% are in bold and >95% are underlined.
c Likelihood ratio test: 2ΔL = 2(lnL_{alternative hypothesis} − lnL_{null hypothesis}).
Figure 7. Deletion Analysis of AVRblb2 Reveals a 34–Amino Acid Region Sufficient for Induction of Rpi-blb2–Mediated Cell Death.

RXLR and deletion mutants of PexRD40170-7 were coexpressed with Rpi-blb2 by agroinfiltration in *N. benthamiana* to determine the AVRblb2 domains required for induction of the Rpi-blb2–mediated HR. A schematic view of the different mutant and deletion constructs is shown on the left. Symptoms of infiltration sites coexpressing the AVRblb2 construct with Rpi-blb2 are shown on the right. HR cell death index with plus and minus signs indicate the presence and absence of effector activity, respectively. The assays were repeated at least three times with similar results. Photograph of symptoms were taken 5 to 7 DAI. SP, signal peptide.
leaves with FLAG antisera revealed no differences in intensity between the four FLAG-PexRD40 proteins (Figure 8C). We conclude that the proteins are equally stable in planta and that the difference in Rpi-blb2–mediated HR cannot be attributed to PexRD40V69F protein instability. Taken together, these results along with the phenotypes observed with the 13 AVRblb2 homologs and the delimitation of the avirulence activity to the 34–amino acid region indicate that the positively selected residue 69 is critical for perception by Rpi-blb2.

**DISCUSSION**

In this study, we employed an effectoromics strategy to perform high-throughput screens for effector activity using a library of 62 candidate RXLR effectors from the potato late blight pathogen *P. infestans*. We were successful in assigning an effector activity to 16 of the assayed 62 proteins, including suppression of cell death, as well as nonspecific and R protein–mediated elicitation of cell death. These results further support the view that functional genomics pipelines can be particularly successful to identify effectors from mined sequence data (Torto et al., 2003; Kamoun, 2006). We increased our success rate by refining the criteria for selecting candidates and focusing only on the RXLR effector class. In addition, we took advantage of the PVX agroinfection method that enables sensitive and high-throughput in planta expression assays by wound inoculation (Takken et al., 2000; Nasir et al., 2005; Takahashi et al., 2007; Vleeshouwers et al., 2008; Bos et al., 2009).

Haas et al. (2009) recently predicted a total of 563 RXLR effector genes, grouped in 149 families, from the genome sequence of *P. infestans* strain T30-4. Our library of 62 clones obtained from 32 primer pairs was generated prior to the completion of the genome sequence and at first glance may appear poorly representative of RXLR effector diversity in *P. infestans*. Nonetheless, we successfully identified two Avr genes as well as novel elicitors and suppressors of cell death and assigned activities to 16 of the 62 effectors. How can such a high success rate be obtained with an apparently underrepresentative library? One explanation is that the majority of the selected genes are expressed because they were mined from *P. infestans* EST data sets (Kamoun et al., 1999a; Randall et al., 2005). Indeed, 27

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**Figure 8.** The Positively Selected Amino Acid 69 of AVRblb2 Is Critical for Activation of Rpi-blb2 Hypersensitivity.

(A) Schematic view of pGR106-PexRD40_{170-7} (AVRblb2) site-directed mutant constructs. FLAG refers to the FLAG epitope tag. V (Val), A (Ala); I (Ile), and F (Phe) refer to the amino acids at position 69 with the top construct (V69) corresponding to PexRD40_{170-7}. The numbers refer to the amino acid positions based on the full-length protein.

(B) Symptoms observed in *N. benthamiana* infiltration sites coexpressing the PexRD40_{170-7} constructs with (+) or without (−) Rpi-blb2. Photographs were taken 6 DAI. *A. tumefaciens* solutions were mixed in a 1:1 ratio before infiltration into *N. benthamiana* leaves. V69, A69, I69, and F69 refer to the constructs described in (A). The negative control was *A. tumefaciens* strains carrying pGR106-GFP (GFP).

(C) In planta accumulation of PexRD40 proteins. A FLAG immunoblot was performed on total protein extracts of leaves of *N. benthamiana* following agroinfiltration with the constructs described in (A). An ∼10-kDa protein band representing recombinant PexRD40 was detected in total extracts of plant tissues expressing all PexRD40 constructs but not the ΔGFP negative control. Equal loading was checked by PonceauS staining.

(D) Percentages of infiltration sites with Rpi-blb2–mediated hypersensitive cell death based on two independent experiments scored at 4 DAI. Error bars indicate SE.

*N. benthamiana* leaves with FLAG antisera revealed no differences in intensity between the four FLAG-PexRD40 proteins (Figure 8C). We conclude that the proteins are equally stable in planta and that the difference in Rpi-blb2–mediated HR cannot be attributed to PexRD40V69F protein instability. Taken together, these results along with the phenotypes observed with the 13 AVRblb2 homologs and the delimitation of the avirulence activity to the 34–amino acid region indicate that the positively selected residue 69 is critical for perception by Rpi-blb2.
(84%) out of our 32 candidates are induced in planta (see Supplemental Table 4 online), whereas of the total RXLR effectors predicted by Haas et al. (2009) only 129 (23%) of the 563 are induced in potato. These results further confirm the observation that selecting candidate effectors from cDNA sequences can be extremely productive even in the absence of a genome sequence (Torto et al., 2003; Tian et al., 2004; Liu et al., 2005). Nonetheless, in the future, an expanded genome-wide collection covering at least all the expressed effectors will provide an even more useful resource.

Suppression of plant innate immunity has emerged as the primary function of bacterial effectors and is likely to be an important activity of oomycete, fungal, and nematode effectors as well (Block et al., 2008; Hogenhout et al., 2009). Nevertheless, our screen of suppressors of cell death response triggered by the PAMP-like secreted protein INF1 revealed only two new effectors in addition to AVR3aK1. These effectors, PexRD8 and PAMP-like secreted protein INF1 revealed only two new effec-
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resource.

The promotion of cell death elicited by PexRD2 could reflect the effector activity of this protein. Ectopic expression of numerous bacterial Type III secretion system effectors (Kjemtrup et al., 2000; Cunnac et al., 2009; Gurlebeck et al., 2009) and P. infestans Crinklers (Torto et al., 2003; Haas et al., 2009) is known to alter host immunity, resulting in tissue necrosis, browning, and chlorosis. In Pseudomonas syringae, 14 TTSS effectors elicit cell death when expressed in N. benthamiana (Cunnac et al., 2009). Additional assays with pGR106-PexRD2 indicated that the observed cell death response is nonspecific and occurs also in the host plant potato as well as 10 additional Solanum species (Vleeshouwers et al., 2008).

The biological relevance of nonspecific cell death promotion by effectors remains ambiguous. One possibility is that promotion of cell death could reflect the virulence function of PexRD2, perhaps as a result of excessive activity on an effector target (Cunnac et al., 2009). This possibility is further strengthened by the emerging view that effectors are promiscuous proteins that bind more than one host target (Van der Hoorn and Kamoun, 2008; Hogenhout et al., 2009). Therefore, the cell death elicitation phenotype could have resulted from aberrant activation of host targets other than the operative target (Van der Hoorn and Kamoun, 2008). In addition, the cell death phenotype could be due to the artificially high expression levels of PexRD2, which is inherent to the A. tumefaciens-based assay. Alternatively, the effectors could trigger the HR in a typical avirulence fashion. This is supported by our finding that PexRD2-mediated cell death is dependent on the ubiquitin ligase-associated protein SGT1 (Figures 4C and 4D), which is required for nucleotide binding site–leucine-rich repeat (NBS-LRR) protein activity (Austin et al., 2002; Azevedo et al., 2002; Peart et al., 2002). However, in side-by-side assays, PexRD2 triggered a much weaker response than the HR elicited by P. infestans AVR proteins or INF1 (Figures 4A and 5A), and the PexRD2 gene is conserved in P. infestans with no evidence of diversifying selection (Table 1). Nonetheless, PexRD2 cell death may have resulted from weak recognition by an N. benthamiana NBS-LRR protein. In such a case, the activity of this NBS-LRR protein must be conserved in other plants, such as potato and tomato, possibly through the recognition of a conserved solanaceous protein targeted by PexRD2.

Vleeshouwers et al. (2008) recently identified AVRblb1 by screening an earlier version of the PexRD library on late blight resistant Solanum genotypes. Here, we independently isolated and confirmed the identity of AVRblb1 as IPIO (PexRD6) using coexpression with S. bulbocastanum Rpi-bb1 in N. benthamiana. In addition, we discovered candidate AVRblb2 (PexRD39/ 40), a previously unknown family of effectors that activate a different S. bulbocastanum gene, Rpi-bb2. These genes trigger Rpi-bb2–specific hypersensitivity following heterologous expression in N. benthamiana, but independent confirmation of their identity as AVRblb2 will require isogenic P. infestans strains with differential virulence.

The finding that some of the Avrblb1 and Avrblb2 alleles are not, or are weakly, recognized by their cognate Rpi-bb gene suggests that they may have evolved to evade recognition by resistant Solanum plants. A degree of coevolution between P. infestans and host plants carrying R genes with Rpi-bb1 and Rpi-bb2 activities is likely. Although S. bulbocastanum is distributed outside the known natural range of wild P. infestans populations, Rpi-bb–like activities were noted in wild Solanum spp that are naturally infected by P. infestans at its center of diversity in Toluca Valley, Mexico (Vleeshouwers et al., 2008); thus, virulent Avrblb alleles may have evolved. With the Avrblb genes at hand, we are now in a position to monitor the potential emergence of virulent races that may accompany the agricultural deployment of the Rpi-bb genes and rigorously assess the broad-spectrum activities reported for Rpi-bb1 and Rpi-bb2 (Helgeson et al., 1998; Song et al., 2003; van der Vossen et al., 2003; Kuhl et al., 2007; Halterman et al., 2008).

Cloning of the Avrblb genes has consequences for understanding the basis of broad-spectrum disease resistance mediated by the Rpi-bb genes. Until recently, the only R genes available to potato breeders have been the R1 to R11 genes originating from S. demissum. However, the usefulness of these R genes proved short-lived because virulent races of P. infestans rapidly emerged following the introduction of resistant potato cultivars (Fry, 2008). Two Avr genes, Avr3a and Avr4 (also termed PAvr4), perceived by S. demissum R3a and R4, respectively, have been identified (Armstrong et al., 2005; van Poppel et al., 2008). Avr4 occurs as a single-copy gene in the P. infestans genome, while Avr3a is the only expressed gene among a small gene family (Armstrong et al., 2005; Haas et al., 2009; van Poppel et al., 2008). Isolates virulent on R3a potatoes carry the allele Avr3aEM, which unlike its counterpart Avr3aEI, is not recognized by R3a (Armstrong et al., 2005). P. infestans isolates virulent on R4 potatoes carry pseudogenized or deleted loss-of-function
alleles of Avr4 (van Poppel et al., 2008). Avrbib1 and Avrblb2 differ from these genes by occurring as expanded gene families with several paralogs targeted by the cognate Rpi-bibb gene. Therefore, multiple independent mutations would be required for P. infestans to become virulent on Rpi-bibb potatoes possibly delaying the emergence of virulent races. In addition, the Avrbib genes are likely important for P. infestans fitness since the pathogen always carries intact coding sequences of these genes. Future functional and population studies, as well as cloning of additional P. infestans Avr genes, will help to identify the features of the Avrbib genes that make them less likely to overcome rapidly their cognate R genes.

AVRblb2 carries a conserved RXLR motif (RSLR) but lacks the dEER sequence that is found in the majority of validated oomycete effectors, confirming that the dEER motif is not absolutely invariant in RXLR effectors (Rehmany et al., 2005; Win et al., 2007). This is surprising because mutations in the dEER motifs of P. sojae AVR1b and P. infestans AVR3a were shown to abolish avirulence in transgenic strains, suggesting that this motif is required for host translocation (Whisson et al., 2007; Dou et al., 2008a). The RXLR-dEER motifs are known to define a host translocation domain of ~25 to 30 amino acids (Bhattacharjee et al., 2006; Whisson et al., 2007; Dou et al., 2008b; Grouffaud et al., 2008). One possibility is that IEAQEVIQSGR, the sequence immediately following the RSLR motif in AVRblb2, is functionally similar to the dEER sequence.

The C-terminal effector region of AVRblb2 that follows the RSLR sequence is only 54 amino acids making it unlikely that AVRblb2 directly performs an enzymatic activity. Most likely, AVRblb2 carries out its virulence and avirulence activities by binding one or more host proteins. At this stage, we cannot rule out that AVRblb2 directly binds Rpi-bibb2, possibly through the 34-amino acid region that is sufficient for activation of hypersensitive cell death. Similar to H. arabidopsidis ATR13 (Allen et al., 2004, 2008) and Melampsora lini AVRRL567 (Dodds et al., 2004, 2006), AVRblb2 displays very high levels of polymorphism (10 polymorphic sites out of 54 in the effector domain) and diversifying selection (up to eight sites under positive selection). How these effectors can be so polymorphic while maintaining their virulence activities remains unclear.

Sequence comparisons of AVRblb2 homologs with differential activities combined with site-directed mutagenesis highlighted residue 69 as critical for recognition by Rpi-bib2. Remarkably, the maximum likelihood method implemented in the codeml program pointed to amino acid 69 as the only positively selected residue when paralogous sequences were used following the strategy of Win et al. (2007). This confirms that positive selection tests on paralogous genes obtained from a single genome sequence can be useful predictors of functionally critical residues (Win et al., 2007).

We observed that the RXLR sequence is not required for cell death induction when a full-length construct containing the native signal peptide is expressed in plant cells (Figure 7) consistent with our previous experiments with AVR3a (Bos et al., 2006). However, these results fail to confirm the findings of Dou et al. (2008a) who showed using a biolistic assay that the RXLR sequence is required for cell death inducing activity when a full-length AVR1b is expressed in soybean cells. We further explored this discrepancy by expressing in N. benthamiana several combinations of sequences that add up to five constructs to assess the effect of different parameters on this experiment. The constructs correspond to (1) three different vectors, including viral and nonviral vectors; (2) three different signal peptides, including signal peptides from the tomato proteins PR1a and P69B; and (3) three different RXLR domains, including P. sojae AVH1b RXLR domain, which is identical to AVR1b (see Supplemental Table 6 online). In all cases, we failed to detect any effect caused by the RXLR to AXAA mutation and equal levels of cell death induction were noted (see Supplemental Table 6 online). In summary, we view these experiments as inconclusive with regards to the ability of RXLR effectors to enter plant cells in the absence of the pathogen. One possible explanation is that the signal peptides are not fully effective and that mis-targeting of the RXLR effectors from the endoplasmic reticulum into the cytoplasm takes place, resulting in intracellular protein accumulation and activation of cell death.

This study is an initial attempt to address the challenge of assigning biological functions to the enormous number of effector genes unraveled by sequencing the P. infestans genome. Here, we further validate the approach of screening effectors by expressing them directly inside plant cells (Torto et al., 2003; Veeshouwers et al., 2008; Guo et al., 2009; Wroblewski et al., 2009). The diverse activities ascribed here to several RXLR effectors support the view that these proteins form a critical class of host translocated effectors in oomycetes. Detailed analyses of the AVRblb2 family revealed a highly polymorphic and complex family in P. infestans and offered insights into the modular structure of this protein. The challenge now is to identify the host targets of effectors like AVRblb2 and understand how these effectors perturb host processes.

METHODS

Microbial Strains, Plants, and Culture Conditions

*Escherichia coli* DH5α and *Agrobacterium tumefaciens* GV3101, GV2260, and AGL0 (Hellens et al., 2000) were routinely grown in Luria-Bertani (LB) media (Sambrook and Russell, 2001) with appropriate antibiotics at 37 and 28°C, respectively. All bacterial DNA transformations were conducted by electroporation using standard protocols (Sambrook and Russell, 2001). *Phytophthora infestans* strains (see Supplemental Table 2 online) were cultured on rye sucrose agar (Caten and Jinks, 1968) at 18°C. For genomic DNA and RNA extractions, plugs of *P. sojae* and *P. infestans* were cultured on rye sucrose agar (Caten and Jinks, 1968) at 18°C. For genomic DNA and RNA extractions, plugs of *P. sojae* mycelium were transferred to modified Pich medium (Kamoun et al., 1993) and grown for 2 weeks before harvesting. *Nicotiana benthamiana* and tomato (*Solanum lycopersicum* cv Ohio 7814) plants were grown and maintained at 22 to 25°C in controlled greenhouse under 16/8-h light-dark photoperiod.

**PexRD Gene Selection and Cloning**

The PexRD genes were mined from a large collection of >80,000 ESTs (Randall et al., 2005). Initially, a set of 50 genes was selected, but this was reduced to 32 genes because 18 genes either failed to fulfill the RXLR effector prediction criteria of Win et al. (2007) or were problematic (poor PCR amplifications, incomplete open reading frames, etc.). Primers corresponding to the 32 candidate RXLR effector genes (see Supplemental Table 1 online) were used in PCR amplification reactions with genomic DNA from 26 *P. infestans* isolates as template (see
Supplemental Table 2 online). None of the examined 32 genes carry introns. The PexRD derivatives were amplified by PCR using the oligonucleotide combinations indicated in Supplemental Table 1 online and then cloned into the Clal and NotI sites of the A. tumefaciens binary PVX vector pGR106 (Lu et al., 2003). The sequences of the pGR106 inserts of the entire collection of PexRD clones are shown in Supplemental Data Set 1 online. A DNA fragment corresponding to 34 amino acids of AVRblb2 (residues 48 to 81) was synthesized by GenScript and inserted into the Pacl and NotI sites of Tobacco mosaic virus binary vector pJL-TRBO (Lindbo, 2007) because its small size prevented cloning into pGR106. All other deletion mutants were obtained by PCR amplifications using appropriate primers (see Supplemental Table 7 online) and were digested with the Clal and NotI restriction enzymes for cloning into the pGR106 vector. As a negative control for the PVX assays, we used the pGR106-ΔGFP construct carrying a truncated and reversed fragment of the GFP gene (Bos et al., 2006). All constructs were verified by sequencing.

RT-PCR Analysis

Time courses of P. infestans infection of detached tomato leaves were performed using zoospore droplet inoculations as described by Kamoun et al. (1998). Discs of equal sizes surrounding the inoculation droplets were dissected from infected leaves and frozen in liquid nitrogen for immediate use or stored at −80°C for later RNA extraction. Total RNA was extracted from infected tomato leaves using the TRIZOL solution (Invitrogen). First-strand cDNA was synthesized using 2 μg of total RNA, oligo (dT) primer, and M-MLV reverse transcriptase (Invitrogen) according to the manufacturer’s instructions. The oligonucleotides used to amplify PexRD transcripts are listed in Supplemental Table 1 online. All primer pairs used for RT-PCR amplified PCR products of the expected size from genomic DNA of P. infestans 88069 and 90128. All RT-PCR amplifications were confirmed using at least a second independent replicate of the infection time course and by comparison to independently published expression analyses of potato (Solamnum tuberosum) infections (Whisson et al., 2007; Haas et al., 2009). Controls consisted of the constitutive ef2α (Torto et al., 2002) and the in planta-induced epitT (Tian et al., 2004).

For RT-PCR analysis in the VIGS experiment, total RNA was extracted from control (dGFP) and SGT1-silenced plants carrying the negative control TRV2-dGFP vector construct, and five plants were inoculated with the SGT1-silenced strain. The inoculated plants were placed in a growth room at 24°C, 60% relative humidity, and a 16/8-h light-dark cycle. A set of 10 plants was used; five plants were inoculated with the control TRV2-dGFP vector construct, and five plants were inoculated with the TRV2-NbSGT1 construct. At day 21 after inoculation, transient coexpression of PexRD2 and p19 (both in GV3101) was performed by mixing the appropriate A. tumefaciens cultures in induction buffer at a ratio of 1:1 (final OD600 of 0.6). Silenced leaves were sampled for RNA extraction for RT-PCR analysis as described above.

Yeast Signal Sequence Trap System

We used the yeast signal trap system based on vector pSUC2TT7M13ORI (pSUC2), which carries a truncated invertase gene, SU2, lacking both the initiation Met and signal peptide (Jacobs et al., 1997). DNA fragments coding for the signal peptides and the following two amino acids of RXLR peptides were inoculated into tomato (Primex Rd, Rd39, and Rd40) were synthesized by GenScript and introduced into pSUC2 using EcoRI and Xhol restriction sites to create in-frame fusions to the invertase (see Supplemental Table 5 online). Next, the invertase negative yeast strain YTK12 (Jacobs et al., 1997) was transformed with 20 ng of each one of the pSUC2-derived plasmids individually using the lithium acetate method (Gietz et al., 1995). After transformation, yeast was plated on CM-Trp plates (minus Trp) on YPRAA plates (1% yeast extract, 2% peptone, 2% raffinose, and 2 μg/mL anticanycin A) containing raffinose and lacking glucose. Also, invertase enzymatic activity was detected by the reduction of TTC to insoluble red-colored triphenylformazan as follows. Five milliliters of sucrose media were inoculated with the yeast transformants and incubated at 30°C for 24 h. Then, the pellet was collected, washed, and resuspended in
calculated the rates of nonsynonymous nucleotide substitutions per previously described by Liu et al. (2005) and Win et al. (2007). We

Avrblb2 Polymorphism Analysis

We used the strategy of Liu et al. (2005) to amplify Avrblb2 from six *P. infestans* isolates using high-fidelity Pfu polymerase (Stratagene). Ampli-

Sequence Analysis

Similarity searches and the majority of the other bioinformatics analyses were performed locally on Mac OSX workstations using standard bioinformatics programs such as BLAST 2.2.11 (Altschul et al., 1997), HMMPer (http://hmmer.janelia.org; Eddy, 1998), ClustalW (http://www.

Positive Selection Analyses

For the positive selection analyses, we closely followed the procedures previously described by Liu et al. (2005) and Win et al. (2007). We calcu-

Immunoblot Analyses

Leaf tissue was harvested 5 DAI, and proteins were extracted as de-

Accession Numbers

Sequence data from this article can be found in GenBank under the following accession numbers: AATU01000000 (*P. infestans* T30-4 genome sequence), GQ869413-GQ869474 (inserts of 62 PexRD clones; see Supplemental Data Set 1 online), and GQ869389-GQ869412 (*Avrblb2* sequences; see Supplemental Data Set 2 online).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. RT-PCR Expression Analysis of PexRD Genes.

Supplemental Figure 2. Besides AVR3a, PexRD8 and PexRD36-1 Suppress the Hypersensitive Cell Death Induced by INF1.

Supplemental Figure 3. Pairwise Comparison of Nucleotide Substitution Rates in 24 AVRblb2 Sequences from Phytophthora infestans.

Supplemental Table 1. Primer Sets Used for Allele Mining, Cloning, and RT-PCR of the PexRD Genes. 

Supplemental Table 2. Phytophthora infestans Isolates Used in This Study.

Supplemental Table 3. PexRD Families.

Supplemental Table 4. PexRD Genes Shown to Be Induced in Potato by Whisson et al. (2007) and Haas et al. (2009). 

Supplemental Table 5. PexRD Signal Peptide Sequences Fused to Invertase in the pSUC2 Vector.


Supplemental Table 7. Primer Sets Used for Cloning of Avrblb2 Deletion Constructs and Their Corresponding Plasmids.

Supplemental Data Set 1. Infection-Ready Collection of 62 Nonre-
dundant Phytophthora infestans RXLR Effectors.

Supplemental Data Set 2. Avrblb2 Sequences Identified in Phytophthora infestans.

Supplemental Data Set 3. Pairwise Comparison of the Ratios (\(\omega = d_s/d_o\)) of Nonsynonymous Nucleotide Substitution (\(d_o\)) Rates and \(d_s\) Values among 24 Avrblb2 Sequences. 

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In planta expression screens of *Phytophthora infestans* RXLR effectors reveal diverse phenotypes, including activation of the *Solanum bulbocastanum* disease resistance protein Rpi-blb2

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