This article is from the December 2006 issue of

Molecular Plant-Microbe Interactions®

published by
The American Phytopathological Society

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Targeted Gene Mutation in *Phytophthora* spp.

Kurt H. Lamour,¹ Ledare Finley,¹ Oscar Hurtado-Gonzales,¹ Daniel Gobena,¹,² Melinda Tierney,³ and Harold J. G. Meijer²

¹The University of Tennessee, Department of Entomology and Plant Pathology, Rm 205 Ellington Plant Science, 2431 Joe Johnson Dr., Knoxville 37996, U.S.A.; ²Laboratory of Phytopathology, Plant Sciences Group, Wageningen University, Binnenhaven 5, NL-6709 PD Wageningen, The Netherlands; ³The University of Tennessee and Oak Ridge National Laboratory, Genome Science and Technology Graduate Program, 1060 Commerce Park, Oak Ridge 37830-8026, U.S.A.

Submitted 22 March 2006. Accepted 12 July 2006.

The genus *Phytophthora* belongs to the oomycetes and is composed of plant pathogens. Currently, there are no strategies to mutate specific genes for members of this genus. Whole genome sequences are available or being prepared for *Phytophthora sojae*, *P. ramorum*, *P. infestans*, and *P. capsici* and the development of molecular biological techniques for functional genomics is encouraged. This article describes the adaptation of the reverse-genetic strategy of targeting induced local lesions in genomes (TILLING) to isolate gene-specific mutants in *Phytophthora* spp. A genomic library of 2,400 ethyl nitrosourea (ENU) mutants of *P. sojae* was created and screened for induced point mutations in the genes encoding a necrosis-inducing protein (*PsojNIP*) and a *Phytophthora*-specific phospholipase D (*PsPXTM-PLD*). Mutations were detected in single individuals and included silent, missense, and nonsense changes. Homozygous mutant isolates carrying a potentially deleterious missense mutation in *PsojNIP* and a premature stop codon in *PsPXTM-PLD* were identified. No phenotypic effect has yet been found for the homozygous mutant of *PsojNIP*. For those of *PsPXTM-PLD*, a reduction in growth rate and an appressed mycelial growth was observed. This demonstrates the feasibility of target-selected gene disruption for *Phytophthora* spp. and adds an important tool for functional genomic investigation.

Additional keywords: knockout, SNP.

The genus *Phytophthora* comprises more than 60 species, many of which are important plant pathogens (Erwin and Ribiero 1996). They belong to the oomycetes, a group of pathogens that have evolved distinct from plants, animals, and fungi (Baldauf 2003; Keeling et al. 2005). Significant resources currently are being devoted to large-scale sequencing and research. Draft genome sequences for the soybean pathogen *Phytophthora sojae* and the sudden oak death pathogen *P. ramorum* became public in 2004, and genome sequences for the late-blight pathogen *P. infestans* and the vegetable pathogen *P. capsici* currently are being generated. The increasing amount of sequence data available for *Phytophthora* spp. prompts the development of genetic tools to explore the functions of novel genes that are identified. Direct strategies to disrupt specific genes have yet to be developed, and indirect methods that rely on random gene disruption using inserted DNA (e.g., knockout libraries with T-DNA or transposable elements) have yet to be accomplished.

Transformation of *Phytophthora* spp. and other oomycete species is technologically challenging and has been successful for only a few species. *Phytophthora* spp. transformed to this point include *P. infestans* (Judelson and Michelmore 1991; Kamoun et al. 1998; Latijnhauwers and Govers 2003; van West et al. 1998), *P. sojae* (Judelson et al. 1993), *P. palmivora* (Vijn and Govers 2003), and *P. parasitica* (Bottin et al. 1999). Thus far, the only *Pythium* spp. transformed are *Pythium ultimum* (Vijn and Govers 2003) and *Pythium aphanidermatum* (Weiland 2003). The techniques utilized for *Phytophthora* spp. transformation include PEG-mediated transformation of protoplasts (Judson and Michelmore 1991), electroporation of zoospores (Latijnhauwers and Govers 2003); projectile bombardment (Cvitianich and Judelson 2003); and Agrobacterium-mediated transformation (Vijn and Govers 2003). Each transformation method has its drawbacks. Producing protoplasts can be difficult and tedious, with PEG-mediated transformations yielding as few as two transformants per microgram of DNA (van West et al. 1998). Bombardment of germinated sporangia gave a higher efficiency (14 transformants/shot/microgram of DNA) compared with PEG-mediated transformation, whereas Blanco and Judelson (2005) found PEG-mediated transformation of protoplasts superior to electroporation. Agrobacterium tumefaciens-mediated transformation has been used successfully to transform *Phytophthora* infestans, *P. palmivora*, and *Pythium ultimum* and produced up to 30 transformants per 10⁷ zoospores (Vijn and Govers 2003).

The ultimate goal of transformation of *Phytophthora* spp. has been to knockout or silence genes of interest to elucidate their function. At this time, very few genes (other than reporter genes *GUS* and *GFP*) have been silenced in *Phytophthora* species. In *Phytophthora infestans*, *inf1* (Kamoun et al. 1998; van West et al. 1999), *cdc14* (Ah Fong and Judelson 2003), *Pibp1* (Blanco and Judelson 2005), *Pgib1* (Latijnhauwers and Govers 2003), and *Pigg1* (Latijnhauwers et al. 2004) are the only genes for which stable gene silencing has been demonstrated. In *P. para-
Sitica, the only other Phytophthora sp. in which silencing has been demonstrated, cbel was silenced by transformation (Gaulin et al. 2002). Transient silencing was demonstrated for cdc14 and inf1 in P. infestans, but the extent of the silencing varied significantly over time (Whisson et al. 2005).

Here, we present a reverse-genetic strategy to recover mutants carrying amino-acid-changing lesions in specific genes for Phytophthora spp. The strategy is adapted from the targeting induced local lesions in genomes (TILLING) approach. TILLING was employed successfully in Arabidopsis, zebrafish, lotus, barley, maize, and Drosophila (Caldwell et al. 2004; Henikoff and Comai 2003; McCallum et al. 2000; Perry et al. 2003; Till et al. 2003; Wienholds et al. 2003; Winkler et al. 2005). The process involves the development and screening of a chemically mutagenized population to isolate individuals carrying induced mutations within a gene of interest. We describe the details of TILLING as it has been adapted to P. sojae, present the initial screening for mutations in two gene targets (PsojNIP and PsPXTM-PLD), and present the preliminary characterization of oospore progeny homozygous for a knockout mutation in PsPXTM-PLD.

RESULTS AND DISCUSSION

The TILLING procedure in Phytophthora spp.

An overview of the TILLING process which is modified for Phytophthora spp., starting with the mutagenesis of swimming zoospore to the final characterization of isolates carrying homozygous mutations of a gene of interest, is depicted in Figure 1. The key steps are i) mutagenesis of Phytophthora zoospores and the conservation of the mutant library and isolation of their genomic DNA (Fig. 1A and B), ii) polymerase chain reaction (PCR) amplification of genes of interest and subsequent screening for induced mutations (Fig. 1C), and, finally iii) production of selfed progeny to obtain homozygous mutants for the gene of interest (Fig. 1D).
Mutant library construction.

Mutants were derived from *P. sojae* strain P6497 (Forster et al. 1994) also used for the genome sequencing project (Jiang et al. 2006; Tyler et al. 2006). To deduce the impact of chemical mutagenesis with ethylnitrosourea (ENU), lethality was determined for a range of concentrations. Exposure of the zoospores to doses of ENU ranging from 0.85 to 8.5 mM ENU resulted in a concentration-dependent increase of lethality (Fig. 2). A limited number (between 384 and 1,000 mutants) of zoospores for each ENU concentration was screened to identify a mutation rate of one mutation per approximately 200 kb (two 96-well plates). Recovery of at least one mutation within one 1-kb gene target per 192 individuals required a concentration of 8.5 mM ENU, which consistently resulted in >90% lethality of the zoospores (Figs. 1A and 2). Therefore, this concentration was chosen for the construction of a mutant library of 2,400 individuals (Fig. 1B).

Target selection and library screening.

Using the TILLING strategy, any segment of the genome that can be amplified as a single PCR product can be screened for induced point mutations. To determine whether the TILLING strategy is functional for *P. sojae*, two genes were chosen: the *P. sojae* necrosis-inducing protein-encoding gene *PsojNIP* (Qutob et al. 2002) and a *Phytophthora*-specific phospholipase D gene (*PsPXTM-PLD*), homologous to the *PiPLD1* gene as described for *P. infestans* (Meijer et al. 2005). One of the key criteria was that developed primers amplified a clear, single, approximately 1-kb PCR product. The amplicon sizes were 994 and 1,004 bp for *PsojNIP* and *PXTM-PLD*, respectively. For the two targets, either the entire gene (*PsojNIP*) or a portion of the coding region (*PXTM-PLD*) was amplified. In the latter case, the TILLING targeted part spanned the region encoding the first catalytic HKD motif and the phosphatidylinositol 4,5-bisphosphate [PI(4,5)P₂]-binding domain (Fig. 3).

Screens were conducted on unpooled DNA, derived directly from ENU-exposed isolates. A typical set of gel images is shown in Figure 4. In other organisms that have TILLING initiatives, genomic DNA from multiple individuals often are pooled to reduce the number of gels which must be run (Colbert et al. 2001). Based on the level of signal for both the full-length and truncated PCR products, pooling between two- and fourfold might be possible.

Characteristics of induced mutations.

For *PsojNIP*, 10 induced mutations were detected in a screen comprising 1,824 mutants. The *PsojNIP* target amplicon is 994 bases and the frequency of induced mutation is 1 per 181 kb of sequence. Two of the mutations are silent, one is upstream of the coding sequence, and seven induce an amino acid substitution (Fig. 5; Table 1). Highly conserved residues within *PsojNIP* have been identified based upon comparison with other members of this large and diverse gene family, known as the Nep1-like proteins (NLPs). The NLPs are known to occur in oomycetes, fungi, and bacteria and trigger cell death and defense in plants (Gijzen and Nurnberger 2006). The most conserved region of all NLPs corresponds to a hepta-peptide motif in the central area of the proteins with the sequence “GHRHDEWE” (Gijzen and Nurnberger 2006). In two of the *PsojNIP* mutations identified, K1857 (D124N) and K3489 (W125R), residues within this motif are substituted (Fig. 5). Other TILLING mutants carry substitutions in amino acids that are less conserved, but nonetheless may affect the activity of the protein (Table 1).

For *PXTM-PLD*, 13 mutations were found in a screen of 1,344 mutants. The *PXTM-PLD* target amplicon is 1,004 bases and the frequency of induced mutation is 1 per 104 kb of sequence. Three of the induced changes are silent; nine mutations results in an amino acid substitution, and one caused a premature stop codon (Fig. 3; Table 2). None of the amino acid changes was located in the catalytic motif. Alignment with other *PXTM-PLDs* showed that most of the substitutions are localized in conserved amino acids (Fig. 3). However, when PXPI-PLDs were taken into account, none of the substituted amino acids was strictly conserved. Therefore, the impact of mutations was based mostly on the type of amino acid substitution (Table 2). An attractive candidate mutation for further research is found in K2870; the mutation causes a premature stop codon positioned at the end of the PI(4,5)P₂-binding domain, behind the first catalytic HKD motif. Therefore, the resulting truncated protein is expected to be nonfunctional (Table 2).

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**Fig. 2.** Response of *Phytophthora sojae* zoospores to treatment with the chemical mutagen ethylnitrosourea (ENU). The percentage of survival, as determined by mycelial growth, is depicted against the concentration of ENU that was used to treat the zoospores.
Recombinant mutant progeny.

Because screening was performed on zoospore-derived isolates, all of the induced mutations are in the heterozygous state. This differs from other organisms (e.g., Arabidopsis), where the TILLING population has been through one round of sexual recombination and individuals carry induced mutations in the heterozygous or homozygous state (Caldwell et al. 2004; Henikoff and Comai 2003; McCallum et al. 2000; Perry et al. 2003; Till et al. 2003; Wienholds et al. 2003; Winkler et al. 2005). Therefore, two mutant isolates, K1857 (PsojNIP) and K2870 (PsPXTM-PLD), one for each of the gene targets, were studied in more detail.

P. sojae is self fertile and produces sexual oospores in single culture. Eighty-two oospore progeny were collected from isolate K1857 and the allelic state was tested using an allele-specific real-time PCR single-nucleotide amplified polymorphism (SNAP) strategy (Fig. 6). The progeny of K1857 had the following genotypes: 15 homozygous wild types, 56 heterozygous, and 11 homozygous mutant progeny. A \( \chi^2 \) analysis indicated that our observed values differed significantly from those expected with Mendelian inheritance (\( \chi^2 = 11.35, 2 \text{ df} \)). Colony morphology and growth rates for the homo- and heterozygous progeny classes were not markedly different and preliminary studies indicated that the mutant progeny were able to cause infection on soybean (details not reported).

For isolate K2870, 22 oospore progeny were collected. The allelic state was tested by direct sequencing because it was not possible to design reliable allele specific primers for the PsPXTM-PLD knockout mutation. The progeny included 2 homozygous wild types, 16 heterozygous, and 3 homozygous mutants. One of the heterozygote isolates, LT2096, appeared to make more oospores than its parent strain. To test whether it was possible to recover a higher number of homozygous mutants’ oospore progeny, we also recovered an additional set of 56 oospore progeny from this isolate. The recovered progeny set of 56 oospores was composed of 3 homozygous wild types, 51 heterozygous, and 2 homozygous mutants. Similar to the ratios observed for the PsojNIP mutation, there was an excess of the heterozygous progeny class, and both progeny sets were significantly different from the ratios expected for simple Mendelian inheritance. Some of the possible reasons for the distorted ratios are that homozygous mutant oospore progeny suffer from the lack of a functional PsPXTM-PLD, there is a leak-through of the parental heterozygous mutant, or that another unidentified mutation is responsible for the unequal numbers.

All 73 of the PXTM-PLD wild-type and heterozygous progeny had normal aerial mycelium morphologically similar to the original parental nonmutant parent isolate and all but four had growth rates similar to the parent type (Fig. 7). In contrast, the five homothallic knockout isolates all had slow growth rates and distinctive appressed mycelium. It remains to be deduced in detail how the growth defect is accomplished via mutation in the PXTM-PLD gene. However, studies in plants and yeast might point to its involvement in cellular dynamics (Dhonukshe et al. 2003; Ella et al. 1996; Gardiner et al. 2001; Rudge et al. 1998; Waksman et al. 1996; Zouwail et al. 2005).

Future work on characterizing the mutants will center on PXTM-PLD activity and function.

Concluding remarks.

This is the first report of a targeted strategy to mutate specific genes in Phytophthora spp. The strengths of this approach are its broad applicability and the ability to recover allelic as well as knockout mutations. A weakness is the effect of other mutations that occur during the process of mutagenesis. For each individual in this P. sojae population, it is estimated that one point mutation is present every 100 to 200 kb. With a genome size of approximately 100 million bases, this means that there are 500 to...
1,000 mutations per individual. The predicted 20,000 genes make up an estimated 40% of the total genome size; therefore, the chance for additional missense or nonsense mutations is clearly a factor. Multiple recombinant mutant progeny homozygous for the induced change must carry the functional change ascribed to the induced mutation (e.g., the phenotype is fully linked to the mutation). Although the number of homozygous mutants recovered thus far is limited, it appears that the mutants carrying the knockout mutation for the PsPXTM-PLD gene have a distinctly altered mycelial growth form compared with the other genotype classes. Even in this case, where the phenotype is strictly inherited with an induced mutation, it still is possible that a tightly linked undetected functional mutation is actually responsible for the observed impact. The only way to be certain is to study another mutant carrying an independent deleterious mutation and confirm the observed results.

Another factor that may impact the results from a TILLING screen strategy is genetic redundancy. The PsojNIP gene is only one member of the NLP family in P. sojae that comprises 58 predicted genes with sustained sequence similarity. However, closer examination indicated that at least half of the NLPs are pseudogenes and only three genes (PsojNIP and two others) are represented by expressed sequence tag hits. Two NLP genes previously were analyzed for expression but no transcripts were detected (Qutob et al. 2002). Analysis of PsojNIP also has shown that this gene is specifically expressed during late stages of plant infection (Jiang et al. 2006; Moy et al. 2004; Qutob et al. 2002). Thus, despite the NLPs composing one of the largest gene families known in P. sojae, the evidence suggests that many of these genes either are not expressed or differentially expressed. This increases the chance that PsojNIP mutants display an as-yet-undetected phenotype. It also underscores the importance of prior genomic investigation to determine the suitability of a gene target for TILLING.

Overall, it appears that TILLING provides a valuable tool for elucidation of gene functions in Phytophthora spp. Improvements in SNP (single nucleotide polymorphism) detection technology and in techniques related specifically to manipulating Phytophthora spp. through the sexual stage will greatly improve our current efficiency. Furthermore, this approach should be readily adaptable to any fungal or fungal-like organism that can be grown in broth culture and that has a spore stage amendable to chemical mutagenesis.

MATERIALS AND METHODS

Strains and growth conditions.

P. sojae strain P6497 (University of California, Riverside, U.S.A.) (Forster et al. 1994), was grown on V8 juice agar (unfiltered) amended with PARP (pimaricin, 25 ppm; ampicillin, 100 ppm; rifampicin, 25 ppm; and pentachloronitrobenzene, 25 ppm) for 9 days at room temperature. Sporangial development was induced by repeated washing of the mycelial colonies (Morris et al. 1998; Morris and Ward 1992) and zoospore release was induced by incubating the plates overnight with sterile water. Zoospores were harvested by filtering through two layers of sterile Kimwipe (Kimberly-Clarke Inc., Roswell, GA, U.S.A.) and the number of zoospores was determined by microscope counting.

Mutagenesis.

ENU (Sigma-Aldrich, Inc., St. Louis) was administered as an 85 mM stock solution in dimethylsulfoxide (DMSO) and stored until usage at –20°C. Mutagenesis was accomplished by incubating 5 or 10 ml of swimming zoospores at a zoospore concentration of 10 zoospores/µl at a final concentration of 8.5 mM ENU for 1 h on a Minilab Roller shaker (Labnet Int., Edison, NJ, U.S.A.). The mutagenesis process was halted by addition of an equal volume of 6% sodium thiosulfate and immediately diluted with clarified PARP V8 broth to 1 zoospore/10 µl. Aliquots (10 µl) of diluted zoospore culture then were arrayed into 384-well plates preloaded with 50 µl of V8-PARP broth and incubated for 7 days at room temperature (approximately 23°C) under laboratory lighting. Parallel control reactions were performed in which the ENU treatment was replaced with water. Percent lethality was determined as the ratio between the

![Fig. 4](image-url)
colonies from plates with ENU-treated zoospores and the number of colonies in the control plates.

**Construction of ENU-treated Phytophthora library.**

Colonies derived from ENU-treated zoospores were transferred individually to 60-mm V8-PARP agar plates. The isolates were grown for at least 3 days at room temperature and divided thereafter for long-term storage or for mycelial growth to isolate genomic DNA. For long-term storage, wells of a 24-well deep-well plate containing 1 ml of V8-PARP broth per well. The 24-well plates were covered with AeraSeal rayon breathable tape (PGC Sciences, Frederick, MA, U.S.A.) and incubated at room temperature without agitation for 7 days. The mycelial mats for each isolate were combined and placed into individual wells of a 2-mL 96-well plate preloaded with three 3-mm glass balls and lyophilized for 48 h in a Labconco stoppering tray dryer system (Labconco Corp., Kansas City, MO, U.S.A.) with the incubation chamber set to 0°C for the first 24 h and 24°C for the second 24 h. The dried mycelium then was disrupted to a fine powder using a Mixer Mill MM 300 (Retsch Inc., Newtown, PA, U.S.A.). The genomic DNA was extracted from the powdered mycelium as reported previously (Lamour and Finley 2006).

**Primer design.**

Primers for TILLING were designed using the codons optimized to detect deleterious lesions (CODDLE) primer design program. In total, five sets of primers automatically were designed for each of the two target genes (PsojNIP and PXTM-PLD). Nonfluorescent primers were tested to determine which primers amplified a single product from P. sojae genomic DNA. Once a set of desired primers was identified, IRDye700 and IRDye800 dye-labeled primers were synthesized and used to screen the P. sojae TILLING library. For screening PsojNIP, the primer sequences PsojNIP-F (IRD700-GATTGCCCCTTTTCTTCGCTTA) and PsojNIP-R (IRD800-GCGCGATTAGCGAACGAGATTCAC) were used. The primer pair PsPXTM-PLD-F (IRD700-GCCATCTCCCAACGCGAAAGTCGAG) and PsPXTM-PLD-R (IRD800-GCAGCCGCAACCTTACCAGT) was used for PsPXTM-PLD screening.

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**Table 1. Summary of induced changes for PsojNIP**

<table>
<thead>
<tr>
<th>Position</th>
<th>Isolate</th>
<th>Nucleotide change</th>
<th>Amino acid change</th>
<th>Amino acid property change</th>
<th>Substitution preference</th>
<th>Conserved in Phytophthora sp.</th>
</tr>
</thead>
<tbody>
<tr>
<td>–19</td>
<td>K2141</td>
<td>A → G</td>
<td>Promoter region</td>
<td>Na</td>
<td>Na</td>
<td>Na</td>
</tr>
<tr>
<td>258</td>
<td>K2338</td>
<td>C → T</td>
<td>G → G</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>265</td>
<td>K2302</td>
<td>G → A</td>
<td>Q → Q</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>284</td>
<td>K3177</td>
<td>C → T</td>
<td>A → V</td>
<td>Nonaliphatic and very small to aliphatic</td>
<td>Neutral</td>
<td>No</td>
</tr>
<tr>
<td>353</td>
<td>K3655</td>
<td>G → A</td>
<td>G → E</td>
<td>Very small, hydrophobic to polar, negative charged</td>
<td>Disfavored</td>
<td>No</td>
</tr>
<tr>
<td>370</td>
<td>K1857</td>
<td>A → G</td>
<td>D → N</td>
<td>Negative charge to small polar</td>
<td>Favored</td>
<td>Yes</td>
</tr>
<tr>
<td>373</td>
<td>K3489</td>
<td>T → A</td>
<td>W → R</td>
<td>Aromatic to nonhydrophobic positive charge</td>
<td>Disfavored</td>
<td>Yes</td>
</tr>
<tr>
<td>424</td>
<td>K2533</td>
<td>A → G</td>
<td>I → V</td>
<td>Aliphatic to small aliphatic</td>
<td>Favored</td>
<td>No</td>
</tr>
<tr>
<td>512</td>
<td>K2600</td>
<td>T → G</td>
<td>I → S</td>
<td>Aliphatic to polar</td>
<td>Disfavored</td>
<td>No</td>
</tr>
<tr>
<td>640</td>
<td>K2973</td>
<td>G → C</td>
<td>D → H</td>
<td>Small negative charge to aromatic positive charge</td>
<td>Disfavored</td>
<td>No</td>
</tr>
</tbody>
</table>

* NA = not applicable.

* Nucleotide position from the predicted start codon of Phytophthora sojae ID 130122 in the VBI microbial database.

**Fluorescent target amplification and nuclease treatment.**

The DNA from the mutant library was diluted from 1:100 to 1:150 in 10 mM Tris (pH 8.0) to a final concentration of between 0.01 and 0.05 ng/µl. The individual PCR reactions contained 50 mM KCl, 10 mM Tris (pH 8.3), 0.4 mM dNTP, 0.12 µM IRDye700-labeled forward primer, 0.08 µM unlabeled forward primer, 0.16 µM IRDye800-labeled reverse primer, 0.04 µM unlabeled reverse primer, 0.1875 units of MasterTaq polymerase (Eppendorf, Westbury, NY, U.S.A.), and 5 µl of diluted genomic DNA (final concentration of between 0.05 and 0.25 ng/µl) in a final volume of 10 µl. The PCR cycling conditions were as follows: 95ºC for 2 min, loop 1 (8 cycles of 94ºC for 20 s, 73ºC decreasing –1ºC per cycle for 30 s, 72ºC for 1 min, ramping to 72ºC at 0.5ºC/s), loop 2 (45 cycles of 94ºC for 20 s, 65ºC for 30 s, 72ºC for 1 min, ramping to 72ºC at 0.5ºC/sec), 72ºC for 5 min, 99ºC for 10 min, and a final heteroduplex formation loop (70ºC for 20 s, decreasing –0.3ºC/cycle for 70 cycles) (Colbert et al. 2001). The PCR reactions were performed using an MJ Research DNA Engine Dyad and Disciple (MJ Research, Hercules, CA, U.S.A.) thermal cycler system.

The PCR products were digested with a member of the S1 nuclease family (designated CEL I) purified from celery (*Apium graveolens*). CEL I cleaves mismatches on either strand on the 3′ side of a single base mismatch in heteroduplex DNA. Enzyme purification was carried out according to a modification of the protocol of Oleykowski and associates (1998) and Yang and associates (2000) described by Till and associates (2003). Appropriate concentrations of CEL I for mismatch cleavage were determined by testing enzyme dilutions on PCR amplicons containing known heterozygous bases. CEL I digestions contained the following components in a 20-µl volume: 10 mM Hepes (pH 7.0), bovine serum albumin at 0.2 µg/ml, 10 mM MgSO4, 0.002% Triton X-100, 10 mM KCl, and the 10-µl volume of the PCR reaction. Digestions were performed at 45ºC for 15 min and terminated by the addition of 5 µl of 75 mM EDTA (pH 8.0) (Colbert et al. 2001). Reactions were frozen at –20ºC or held at 4ºC briefly until purification.

**Detection of induced mutations.**

The CEL I-treated PCR fragments were purified using Sephadex G-50 medium columns in Whatman Unifilter 800 96-well spin plates (Whatman Inc., Clifton, NJ, U.S.A.). To prepare the columns, 750 µl of water-hydrated Sephadex G-50 slurry was loaded into each well of the 96-well spin plate and spun at 750 × g for 2 min. The PCR reactions were loaded and spun 2 min at 750 × g into a 96-well PCR plate containing a stop solution/loading dye (37% [vol/vol] formamide, 4 mM EDTA [pH 8.0], and bromophenol blue at 90 µg/ml). The samples were heated uncovered but protected from light at 85ºC for 45 min to reduce the volume prior to loading.

Approximately 1 µl of the reduced product was loaded onto a 100-tooth paper comb (The Gel Company, San Francisco, U.S.A.) utilizing a comb loader (The Gel Company). The paper comb was loaded onto a denaturing 6% acrylamide gel (25 cm) and electrophoresis performed at 1,500 V, 40 mA, 40 W.

**Table 2. Summary of induced changes for PXTM-PLD**

<table>
<thead>
<tr>
<th>Positionb</th>
<th>Isolate</th>
<th>Nucleotide change</th>
<th>Amino acid change</th>
<th>Amino acid property changesc</th>
<th>Substitution preferencec</th>
<th>Conserved in other phospholipase Ds</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,319</td>
<td>K1891</td>
<td>A → C</td>
<td>L to F</td>
<td>Aliphatic to aromatic</td>
<td>Neutral</td>
<td>Yes</td>
</tr>
<tr>
<td>2,351</td>
<td>K2883</td>
<td>C → T</td>
<td>P to L</td>
<td>Changed to aliphatic and hydrophobic</td>
<td>Disfavored</td>
<td>No</td>
</tr>
<tr>
<td>2,410</td>
<td>K2476</td>
<td>T → G</td>
<td>L to V</td>
<td>Aliphatic to small aliphatic</td>
<td>Favored</td>
<td>No</td>
</tr>
<tr>
<td>2,558</td>
<td>K2225</td>
<td>T → C</td>
<td>S to S</td>
<td>NA</td>
<td>Valine in <em>Phytophthora infestans</em></td>
<td>NA</td>
</tr>
<tr>
<td>2,679</td>
<td>K2675</td>
<td>C → T</td>
<td>I to I</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>2,685</td>
<td>K2668</td>
<td>T → A</td>
<td>D to E</td>
<td>Small negative charge to negative charge</td>
<td>Favored</td>
<td>No</td>
</tr>
<tr>
<td>2,745</td>
<td>K2902</td>
<td>C → T</td>
<td>F to F</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>2,753</td>
<td>K2239</td>
<td>T → C</td>
<td>V to A</td>
<td>Aliphatic to nonaliphatic and very small</td>
<td>Neutral</td>
<td>Yes</td>
</tr>
<tr>
<td>2,893</td>
<td>K2870</td>
<td>A → T</td>
<td>K to stop</td>
<td>Polar to stop</td>
<td>Neutral</td>
<td>No</td>
</tr>
<tr>
<td>2,900</td>
<td>K2598</td>
<td>A → T</td>
<td>D to V</td>
<td>Polar to aliphatic; hydrophilic to hydrophobic and charged to neutral</td>
<td>Disfavored</td>
<td>No</td>
</tr>
<tr>
<td>2,991</td>
<td>K2005</td>
<td>G → A</td>
<td>G to E</td>
<td>Aliphatic to polar and neutral to negative charge</td>
<td>Disfavored</td>
<td>No</td>
</tr>
<tr>
<td>3,070</td>
<td>K2248</td>
<td>G → A</td>
<td>V to I</td>
<td>Small aliphatic to aliphatic</td>
<td>Favored</td>
<td>No</td>
</tr>
<tr>
<td>3,083</td>
<td>K1897</td>
<td>C → T</td>
<td>S to L</td>
<td>Polar to aliphatic and hydrophilic to hydrophobic</td>
<td>Disfavored</td>
<td>No</td>
</tr>
</tbody>
</table>

* a NA = not applicable.
* b Nucleotide position from the predicted start codon of *Phytophthora sojae* ID 130122 in the VBI microbial database.
* c According to Betts and Russell (2003).
containing a mixture of oospores and hyphal fragments, was treated with crude lysing enzymes from *Trichoderma harzianum* (Sigma-Aldrich Inc.) at 1 mg/ml for 16 to 20 h with gentle agitation and then diluted to 50 ml with sterile water. The treated oospores were incubated under continuous fluorescent light and checked daily for the presence of germinating oospores. Germinating oospores were harvested using a modified Pasteur pipette as described previously (Lamour and Hausbeck 2000) and transferred to V8-PARP agar plates for growth.

The allelic state of each oospore progeny was tested using direct sequencing or allele-specific PCR. In the latter case, protocols and tools were used that are available on the Ausubel Laboratory web page to design SNAP primers specific to the wild-type and mutant alleles (SNAPER Program). The SNAP assay includes the intentional incorporation of a mismatched base at the 3’ end of an allele-specific forward primer three to four bases from the 3’ end of the primer. Two sets of primers, one for each of the alleles to be assayed, are utilized and, under the described PCR conditions, amplification occurs only in the presence of the correct allele (Drenkard et al. 2000). Four sets of reference and alternative allele primer pairs were tested for each SNP. Primer sets which provided unambiguous results for the reference (Ref. Forward Primer: ACAACGCAGCCTCCGGTAC and Ref. Reverse Primer: AGCCGCGTTATACGACAG) and the alternative (Alt. Forward Primer: ACAA CGCAGCGGCTCCGTTGTT and Alt. Reverse Primer: AGCCG TAGGTTATCAACACG) allele at 28 cycles were chosen to score the oospore progeny.

The SNAP protocol was modified for use in a SYBR Green real-time detection system. SYBR Green Master Mix (Applied Biosystems, Foster City, CA, U.S.A.) was substituted for the Taq polymerase, buffer, and nucleotides and the reactions were run on an ABI PRISM 7000 Sequence Detection System (Applied Biosystems) with the following program: 50°C for 2 min, 94°C for 5 min, and 28 cycles of 94°C for 30 s and 62°C for 1 min. The Ct (cycle threshold) values for the reference and alternate primer pairs were used to score the oospore progeny alleles in place of agarose electrophoresis. An agarose gel with results from screening progeny for mutant isolate K1857 is shown in Figure 6.

**ACKNOWLEDGMENTS**

H. Meijer is financially supported by a grant from the Dutch Ministry of Agriculture, Nature, and Food Quality (LNV427). This material is based upon work supported by the National Science Foundation under grant no. 0347624 to K. Lamour and in part by the National Science Foundation Research Collaboration Networks in Biological Sciences program, grant no. EF-0130263. We wish to thank S. Henikoff, L. Comai, and B. Till at the Seattle Tilling Project for training on TILLING and M. Gijzen, D. Qutob, and B. Tyler for gene sequences and helpful advice as we developed gene targets. We also thank members of the *Phytophthora* Molecular Genetics Network for valuable suggestions and encouragement.

**LITERATURE CITED**


