TECHNICAL ADVANCE

Targeted Gene Mutation in *Phytophthora* spp.

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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 ethylnitrosourea (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 technically 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; Latijnhouwers 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 (Judelson and Michelmore 1991), electroporation of zoospores (Latijnhouwers and Govers 2003), projectile bombardment (Cvitanich 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 with 10⁶ sporangia) but resulted in heterokaryons (Cvitanich and Judelson 2003). Electroporation of protoplasts and zoospores has been used to transform oomycetes with varying results. Latijnhouwers and Govers (2003) reported higher efficiency of transformation with electroporation of zoospores 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*, *infl* (Kamoun et al. 1998; van West et al. 1999), *cdc14* (Ah Fong and Judelson 2003), *Pibzp1* (Blanco and Judelson 2005), *Pigpb1* (Latijnhouwers and Govers 2003), and *Pigpal* (Latijnhouwers et al. 2004) are the only genes for which stable gene silencing has been demonstrated. In *P. para-*

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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 *infl1* 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 tar-

gets (*PsojNIP* and *PsPXTM-PLD*), and present the preliminary characterization of oospore progeny homozygous for a knock-out 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).

Fig. 1. Overview of the targeting induced local lesions in genomes (TILLING) process for *Phytophthora*. **A**, Swimming zoospores are treated with ethylnitrosourea (ENU) and the surviving spores are arrayed into 384-well plates. **B**, Individual mutant colonies are transferred to solid V8 juice agar, assigned a unique identifier, transferred to long-term hemp seed storage, and transferred to 24-well broth plates for mycelium production. Genomic DNA is extracted from the mycelium to create a genomic DNA library. **C**, The genomic DNA library is screened to identify mutants carrying induced mutations within specific genes. Screening is accomplished by i) amplifying a 1 kb target with 700 and 800 nm IRD-labeled forward and reverse primers, respectively; ii) heating and cooling the polymerase chain reaction (PCR) products to form heteroduplexes (HD); iii) treating the resulting HDs with the single-strand-specific nuclease CEL I which cuts 3' of single base mismatches; iv) resolving the treated products on a LiCor sequencing machine; v) identifying isolates carrying induced point mutations; and (vi) characterizing the mutations. **D**, Isolates carrying what appear to be disruptive mutations are taken through the sexual stage to recover recombinant progeny and the homozygous mutant genotypes are assayed to determine the impact of the induced mutation.

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 "GHRHDWE" (Gijzen and Nurnberger 2006). In two of the *PsojNIP* mutants 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-PLD*s showed that most of the substitutions are localized in conserved amino acids (Fig. 3). However, when *PXPH-PLD*s 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).

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 χ^2 analysis indicated that our observed values differed significantly from those expected with Mendelian inheritance ($\chi^2 = 11.35$, 2 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 oo-

spore 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

Fig. 3. Schematic representation of the domain structure of the *Phytophthora sojae* PXTM-PLD protein (PLD_163010). The bar indicates the size of 100 amino acids. The enlarged area in the box has been targeted for targeting induced local lesions in genomes (TILLING). The region is aligned on amino acid level with PXTM-PLD proteins from other *Phytophthora* spp.: *P. infestans*, PiPLD1, AY929154; and *P. ramorum*, PLD_101442. The conserved region around the HKD motif and the PI(4,5)P₂ binding domain are shaded. The HKD motif is underlined. Numbers indicate the amino acid position for each protein. Amino acid changes in *P. sojae* PXTM-PLD that were generated by TILLING are shown with an up-pointing arrowhead over the exact residue. The asterisk indicates the full stop.

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 ma-

nipulating *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 amenable 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. Representative LiCor gel images showing CEL I-treated polymerase chain reaction (PCR) products for a 1-kb portion *PXTM-PLD* gene in *Phytophthora sojae*. The full-length PCR product is visible as a dark line at the top of each of the 96 wells. Amplicons harboring induced mutations are cut into two fragments labeled with either the 700- or 800-nm IR dye. **A**, The 800-nm gel image and the black arrows point to lanes with three novel fragments of approximately 900 nucleotides (nt) (1), 360 nt (2), and 515 nt (3) in length. **B**, The 700-nm image of the same gel and the complementary fragments are clearly visible for the number 2 and 3 mutations and not visible for the much smaller (approximately 100-nt) fragment of mutation 1. The ladder has fragments of 700, 650, 600, 565, 530, 500, 495, 460, 400, 364, 350, 300, 255, 204, 200, 145, 100, and 50 nt.

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, two 7-mm plugs of actively expanding mycelium were transferred to 2-ml screw-cap tubes containing two sterile hemp seed and 1 ml of sterile MilliQ water. For mycelium production, wefts of aerial mycelium were transferred to 4 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 Scientifics, 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., Newton, 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-GATTGCCCCGCC TTTTCTTGCTTA) and *PsojNIP*-R (IRD800-GCGCGATTAG CGAACGAGATTCAC) were used. The primer pair *PsPXTM-PLD*-F (IRD700-GCCATCTCCAACGCCAAGTACGAG and *PsPXTM-PLD*-R (IRD800-GCACGCAACACTTCACCATCG TCT) was used for *PsPXTM-PLD* screening.

Fig. 5. Protein alignment of homologous necrosis-inducing proteins (NIPs) from different *Phytophthora* and *Pythium* spp. Mutated amino acids identified by targeting induced local lesions in genomes (TILLING) are shown with an up-pointing arrowhead over the exact residue in the NIP from *Phytophthora sojae*. Underlined residues correspond to the predicted signal peptide. *P. sojae*: AAM48170, *P. parasitica*: AAK19753, *P. infestans*: AAK25828, *P. megakarya*: AAX12401, *Pythium aphanidermatum*: AAD53944.

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

Position ^b	Isolate	Nucleotide change	Amino acid change	Amino acid property change ^c	Substitution preference ^c	Conserved in <i>Phytophthora</i> sp.
–19	K2141	A → G	Promoter region	NA	NA	NA
258	K2338	C → T	G to G	NA	NA	NA
265	K2302	G → A	Q to Q	NA	NA	NA
284	K3177	C → T	A to V	Nonaliphatic and very small to aliphatic	Neutral	No
353	K3655	G → A	G to E	Very small, hydrophobic to polar, negative charged	Disfavored	No
370	K1857	A → G	D to N	Negative charge to small polar	Favored	Yes
373	K3489	T → A	W to R	Aromatic to nonhydrophobic positive charge	Disfavored	Yes
424	K2533	A → G	I to V	Aliphatic to small aliphatic	Favored	No
512	K2690	T → G	I to S	Aliphatic to polar	Disfavored	No
640	K2973	G → C	D to H	Small negative charge to aromatic positive charge	Disfavored	No

^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).

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: 95°C for 2 min, loop 1 (8 cycles of 94°C for 20 s, 73°C decreasing –1°C per cycle for 30 s, 72°C for 1 min, ramping to 72°C at 0.5°C/s), loop 2 (45 cycles of 94°C for 20 s, 65°C for 30 s, 72°C for 1 min, ramping to 72°C at 0.5°C/sec), 72°C for 5 min, 99°C for 10 min, and a final heteroduplex formation loop (70°C for 20 s, decreasing –0.3°C/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 albumen 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 45°C 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 –20°C or held at 4°C 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 85°C 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*^a

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

^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).

Fig. 6. SNP genotypes for 12 oospore progeny of *Phytophthora sojae* resolved using single-nucleotide-amplified polymorphism (SNAP) primers specific to either C (lanes 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, and 25) or T (lanes 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, and 24). The parent isolate was heterozygous for CT at a site in the *P. sojae* necrosis-inducing protein (*PsojNIP*) gene. Polymerase chain reaction products for the T- and C-specific primers from individual isolates are loaded in adjacent lanes from left to right (vertical lines separate individual isolates) and the resulting genotypes are specified. Lane 1 = 1-kb ladder.

Fig. 7. Colony morphology of the wild-type *Phytophthora sojae* isolate P6497, and representatives of oospore progeny derived from the heterozygous mutant K2870 after 9 days of growth in the dark on full-strength V8 agar. **A**, P6497: wild type; **B**, LT2489: heterozygous (Wt/mt); **C**, LT2938 and **D**, LT2662: homozygous wild-type progeny (Wt/Wt); **E**, LT2506 and **F**, LT2501 homozygous mutant (mt/mt).

and 50°C in 1× Tris-borate EDTA buffer on a LI-COR 4300 DNA analysis system (LI-COR, Lincoln, NE, U.S.A.). Gels were run 3.0 to 3.5 h. Following electrophoresis, TIFF images of the 700 and 800 channels were analyzed using Adobe Photoshop (version 8.0; Adobe Systems Inc., Seattle, WA, U.S.A.) and samples containing DNA fragments in addition to the wild-type PCR product were identified. The size of the additional bands in the 700 and 800 channels provides the approximate location of the SNP in the PCR fragment (± 20 bp) (Fig. 4). The PCR was repeated for mutants carrying induced mutations using unlabeled primers, and the PCR fragments were sequenced. The resulting traces were examined manually using Sequencher (version 4.1.4; Gene Codes Corp., Ann Arbor, MI, U.S.A.) and the presence of the induced change verified. Subsequently, the predicted impact on the coding sequence was assessed.

Recovery of homozygous mutants.

Mutants carrying amino-acid-changing mutations were recovered from long-term storage and grown on 60 mm of V8-PARP agar plates in the dark for 2 months. The mycelial mats on the surface of the plates was scraped off and homogenized thoroughly in 20 ml of sterile water using a Tissue-tearor (Fisher Scientific Inc, Hampton, NH, U.S.A.) for 3 min at 30,000 rpm and filtered through one layer of sterile Kimwipe. The filtrate, 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: ACAACGCACGCCT CCCGGTC and Ref. Reverse Primer: AGCGCCAGCGTTATC AACCACG) and the alternative (Alt. Forward Primer: ACAA CGCAGCCTCCCGGTT and Alt. Reverse Primer: AGCGC CAGCGTTATCAACCACG) allele at 28 cycles were chosen to screen 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.

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AUTHOR-RECOMMENDED INTERNET RESOURCES

CODDLE primer design website: <http://www.proweb.org/input/>
 Massachusetts General Hospital Department of Molecular Biology, Ausubel Lab's SNAPER website: genetics.mgh.harvard.edu/ausubelweb/
 Seattle TILLING Project website: tilling.fhcrc.org:9366/
 VBI Microbial Database
 Virginia Bioinformatics Institute VBI Microbial database: phytophthora.vbi.vt.edu