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I am submitting herewith a thesis written by Melinda Beth Tierney entitled "Reverse Genetics with TILLING in *Phytophthora sojae*." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Life Sciences.

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Reverse Genetics with TILLING in

Phytophthora sojae

A Thesis presented for the Master of Science Degree

The University of Tennessee, Knoxville

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DEDICATION

This thesis is dedicated to John Tierney for always believing in me, for his invaluable support and encouragement, and most of all his love

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ABSTRACT

The reverse genetic method TILLING (Targeting Induced Local Lesions in Genomes) is being applied to the plant pathogen *Phytophthora sojae*. The objective is to recover gene-specific mutants carrying allelic series and/or knockout induced mutations. A library of 3000 mutant individuals was generated using the chemical mutagens ethylmethanesulfonate (EMS) or ethylnitrosourea (ENU). Gene-specific induced mutations are detected by screening a mirror library of genomic DNA. PCR is used to amplify and fluorescently label 1kb portions of specific genes from the mutant library and the PCR products are then heated and cooled slowly to form hetero- and homo-duplexes. The PCR fragments are treated with the CEL 1 enzyme, a single strand specific endonuclease, and the treated fragments resolved on a LI-COR gel analyzer system. Amplicons harboring induced SNPs are cleaved at the site of the mutation thus producing novel fragments. To date we have identified nine SNP's in the Necrosis Inducing Protein (NIP) gene in *P. sojae* using TILLING. Although beyond the scope of this work, the generation of recombinant sexual progeny to recover mutants homozygous for these mutations is underway.

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1. INTRODUCTION

This chapter is a lightly revised version of a paper by the name "An Introduction to Reverse Genetic Tools for Investigating Gene Function" published on the American Phytopathological Society educational website:

Tierney, M.B. and Lamour, K.H. 2005. An Introduction to Reverse Genetic Tools for Investigating Gene Function. *The Plant Health Instructor*. DOI: 10.1094/PHI-I-2005-1025-01.

My primary contributions to this paper include literature review and research of topic information and most of the writing.

A. Genetics

Genetics began in the 1860's with Gregor Mendel, an Augustinian monk who performed experiments that suggested the existence of genes (Griffiths et al. 2000). The discovery of the structure of DNA by James Watson and Francis Crick in the 1950's (Watson and Crick 1953), followed by the development of di-deoxy terminator sequencing by Sanger in the late 1970's (Sanger, Nicklen, and Coulson 1977) and then of PCR (polymerase chain reaction) technology by Kary Mullis in the 1980's (Mullis and Faloona 1987; Saiki et al. 1985) set off a genetic revolution. Technological advances in sequencing have greatly accelerated our accumulation of genetic sequence data to the point now where whole genome sequences are publicly available for a large number of organisms including plant pathogens. The toolbox for genetic research is expanding rapidly and this overview presents a suite of tools generally referred to as reverse genetics that can be used to investigate gene function.

B. Forward Genetics vs. Reverse Genetics

Variants help us understand the 'normal'. Variation can be measured at many scales – from macro (body size, morphology) to different levels of micro variation (crude

protein profiles to DNA sequence variation). Forward genetics refers to a process where studies are initiated to determine the genetic underpinnings of observable phenotypic variation. In many cases the observable variation has been induced using a DNA damaging agent (mutagen) but also may be naturally occurring. The investigator eventually ends up sequencing the gene or genes thought to be involved (Figure 1).

With the advent of whole genome sequencing many researchers are now in a very different position. They have access to all of the gene sequences within a given organism and would like to know their function. So, instead of going from phenotype to sequence as in forward genetics, reverse genetics works in the opposite direction – a gene sequence is known but its exact function is uncertain. In reverse genetics a specific gene or gene product is disrupted or modified and then the phenotype is measured (Figure 1). Here we will overview some of the techniques for reverse genetics with a special emphasis on the TILLING (Targeting Induced Local Lesions IN Genomes) technique which is being utilized on plant pathogens in the genus *Phytophthora*.

C. Specific Reverse Genetic Approaches

The goal in reverse genetics is to investigate the impact of induced variation within a specific gene and to infer gene function. The process of disruption or alteration can either be targeted specifically as in the case of gene silencing or homologous recombination or can rely on non-targeted random disruptions (e.g. chemical mutagenesis, transposon mediated mutagenesis) followed by screening a library of individuals for lesions at a specific location. Following is a brief overview of some commonly used targeted and non-targeted approaches.

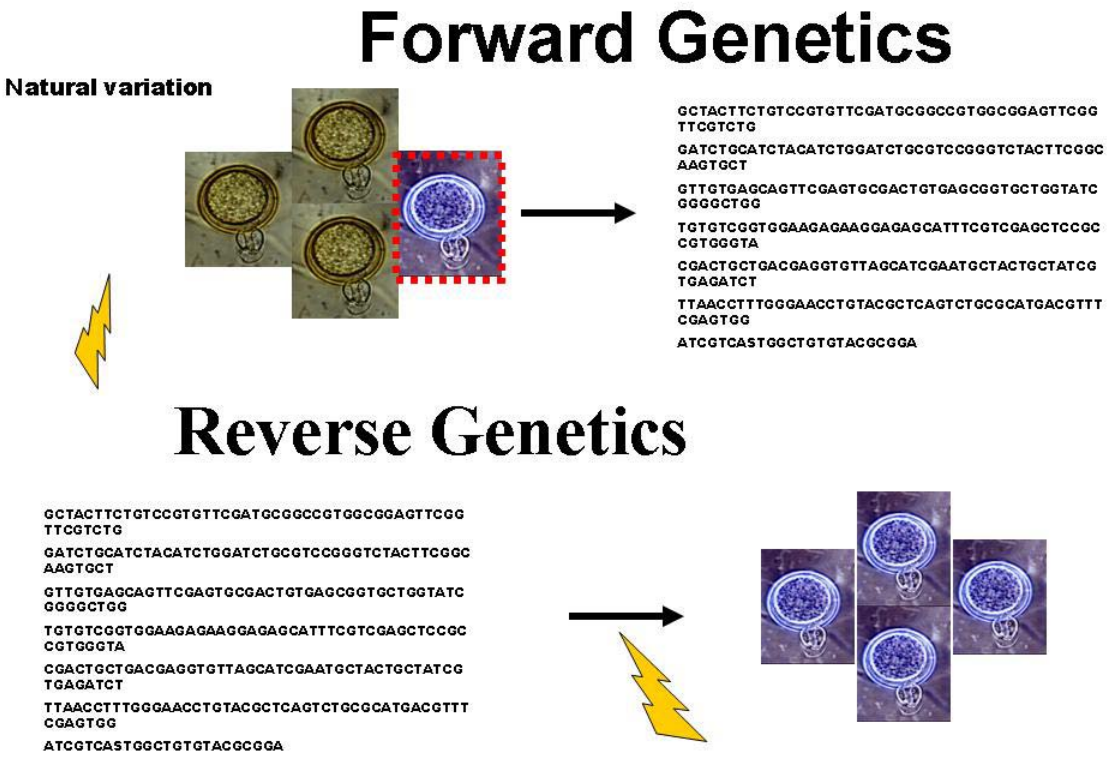


Figure 1: Illustration of the major differences between forward and reverse genetics. The blue oospore symbolizes an observable phenotype that may have been produced via any one of a number of pathways (e.g. mutation, recombination, etc.). This is the starting point for A, forward genetics where the investigator proceeds from the phenotype to characterize the underlying genetic difference. B, illustrates reverse genetics where the sequence for a gene is known but the gene's function is unknown and the investigator disrupts the gene and investigates the resulting phenotype.

C.1. Gene Silencing

RNA interference (RNAi) is the process by which expression of a target gene is inhibited by antisense and sense RNAs. It works based on the ability of double-stranded sequences to recognize and degrade sequences that are complementary to them (Lewin 2004). RNAi was first discovered in *Caenorhabditis elegans* when the introduction of double-stranded RNA was observed to be an efficient method for silencing gene expression (Fire et al. 1998; Kuttenkeuler and Boutros 2004). RNAi- based silencing is an exciting strategy for reverse genetics (Waterhouse, Graham, and Wang 1998). RNA interference has recently become a powerful tool to silence the expression of genes and analyze their loss-of-function phenotype, allowing analysis of gene function when mutant alleles are not available. Having been shown to work in a similar manner in all metazoans, RNAi has proven to be applicable to many organisms and has been used to generate a wide variety of loss-of-function phenotypes (Kuttenkeuler and Boutros 2004). The phenomenon of post-transcriptional gene silencing observed in plants may also be due to a related RNAi mechanism (Waterhouse, Graham, and Wang 1998). RNAi based silencing utilizes the endonuclease Dicer to cleave single stranded RNAs, abbreviated siRNAs, from double stranded RNA; the RISC complex then destroys specific target mRNAs based on sequence complementarity with the siRNA (Pattanayak et al. 2005).

RNAi has been used for a systematic analysis of gene function in *C. elegans* by generating loss of function phenotypes, creating a library of worms expressing dsDNA corresponding to different genes (Lewin 2004). Genome-wide RNAi screens against these libraries of predicted genes have allowed study of a variety of biological processes in *C. elegans*. A genome-wide library of double-stranded RNAs that target every gene in

the *Drosophila* genome has also been published that is suitable for high throughput cell-based assays (Kuttenkeuler and Boutros 2004). One difficulty in using RNAi as a reverse genetic technique is that throughput is limited by the ability to deliver siRNAs to target loci (Henikoff, Till, and Comai 2004). It is also labor intensive, can give ambiguous results, and can be unsuitable for isolating mutants that have lethal or sterile phenotypes (Gilchrist and Haughn 2005).

C.2. Targeted Gene Disruption by Homologous Recombination

Homologous recombination is a reciprocal exchange of DNA sequences, as in between two chromosomes that carry the same genetic loci (Lewin 2004). Just as homologous recombination has been found to be mainly initiated with a double-strand break, gene targeting by homologous recombination is associated with the repair of double strand breaks. The double-strand break repair and synthesis-dependent strand-annealing models are the most generally accepted models to explain gene targeting (Iida and Terada 2004).

Homologous recombination has been widely used in embryonic stem (ES) cells in mice, and has allowed construction of precise mutations in nearly every gene. A reverse-genetic system using homologous recombination has recently been developed for *Drosophila*. It is promising, but is a lengthy procedure and requires generation of specific transgenic flies (Stemple 2004). Reproducible gene targeting by homologous recombination is now also feasible in rice. With the combination of site-specific recombination systems (such as Cre-lox), the future of gene targeting by homologous recombination as a routine procedure for engineering the genome of rice and presumably other plants is bright (Iida and Terada 2004).

C.3. Insertional Mutagenesis/Transposon Mediated Mutagenesis

Transposons are mobile genetic elements that can relocate from one genomic location to another (Hayes 2003). They are DNA sequences that can insert themselves at a new location in the genome without having any sequence relationship with the target locus (Lewin 2004). Transposon-based signature-tagged mutagenesis has been successful in identifying essential genes as well as genes involved in infectivity of a variety of pathogens. Strategies for insertional mutagenesis using transposons have been developed for a number of animal and plant models (Hayes 2003). Reverse-genetics is currently being done in *Drosophila* and *C. elegans* by utilizing libraries of individuals who carry transposable element insertions, many of which have been mapped, and some of which will disrupt the expression of nearby genes. In *Drosophila* P-elements, imprecise excision can be driven to generate a mutation in the nearest gene. Transposon-based methods are also being used in *Arabidopsis*, maize and other plants (Stemple 2004). One drawback of insertional mutagenesis is the low frequency of mutations, necessitating the screening of large numbers of individuals to find mutations in any given gene (Gilchrist and Haughn 2005). Also, insertions in essential genes will usually cause lethality, and less severe mutations must be generated in these genes in order to understand gene function (Till et al. 2003).

The segment of the Ti plasmid of *Agrobacterium tumefaciens* known as T-DNA that carries genes to transform the plant cell has also been utilized for insertional mutagenesis. T-DNA insertional mutagenesis has been used to obtain gene knockouts for greater than 70% of *Arabidopsis* genes (Alonso et al. 2003), but no comparable resources exist for rice or maize even as high-coverage genomic sequence is becoming increasingly

available (Henikoff, Till, and Comai 2004). Unlike other successful gene targeting systems (namely mouse, *Physcomitrella*, and *Drosophila*), the precise mechanism of T-DNA integration into the plant genome remains largely unknown (Iida and Terada 2004). Like RNA suppression techniques, insertional mutagenesis is limited by its host range and by its limited range of allele types (McCallum et al. 2000).

Gene replacement via transformation is a commonly used tool for many filamentous fungi (Fang, Hanau, and Vaillancourt 2002; Lalucque and Silar 2004; Takano et al. 2000). The transformation can be mediated in many ways, including *Agrobacterium* (Zhang et al. 2003) and various other transformation vectors (Scott-Craig et al. 1998; Takano et al. 2000).

D. Chemical Mutagenesis and TILLING

Two of the most widely used mutagens for chemical mutagenesis experiments are EMS (ethylmethanesulfonate) and ENU (ethylnitrosourea). EMS (Sciences 2002; Sega 1984)is a chemical mutagen that alkylates guanine bases. The alkylated guanine will then pair with thymine instead of the preferred cytosine base, ultimately resulting in a G/C to A/T transition. EMS is the most commonly used mutagen in plants. In *Arabidopsis*, five percent of EMS-induced mutations in targeted coding regions result in premature termination of the gene product, while fifty percent result in missense mutations that alter the amino-acid sequence of the encoded protein (Gilchrist and Haughn 2005). This high level of missense mutations relative to terminated gene products is very useful in analyzing gene function. ENU (Justice et al. 1999) also induces point mutations, and is a more potent mutagen than EMS. It is also an alkylating agent, mutagenizing by transferring an ethyl group to oxygen or nitrogen radicals in the

DNA molecule, which leads to mispairing and ultimately results in base pair substitutions, and sometimes base pair losses if not repaired (Guénet 2004).

Chemical mutagenesis is attractive for reverse genetics because it results in induced point mutations, which create a diverse range of alleles for genetic analysis. It induces a large number of recessive mutations per genome that are randomly distributed (Gilchrist and Haughn 2005). Because chemical mutagenesis is already widely used in many organisms for forward genetic screens (Smits et al. 2004), it promises to be generally applicable for reverse genetics (Coghill et al. 2002; Henikoff, Till, and Comai 2004; Jansen et al. 1997; Wienholds et al. 2002). Until recently, chemical mutagenesis has not been widely used as a tool for reverse genetics because of the lack of high-throughput techniques for detecting point mutations (Gilchrist and Haughn 2005). The TILLING strategy provides a high throughput strategy to detect single base changes within genetic targets (Colbert et al. 2001; Henikoff and Comai 2003) and can be applied to a wide variety of organisms (McCallum et al. 2000; Perry et al. 2003; Smits et al. 2004; Till, Reynolds et al. 2004).

E. Examples of TILLING

TILLING has been an instrumental tool in the study of several organisms. Following is the description of two of the most widely recognized TILLING projects.

E.1. *Arabidopsis*

A public TILLING resource was set up in 2001 for the *Arabidopsis* community. This effort was called the *Arabidopsis* TILLING Project (ATP) and is now called the Seattle Tilling Project (STP) and is a joint effort between the Comai Laboratory at the University of Washington and the Henikoff Laboratory at the Fred Hutchinson Cancer

Research Center in Seattle, Washington. Users are charged a fee that covers partial costs of the services provided to request mutations in genes of interest (Gilchrist and Haughn 2005; Till et al. 2003). Training sessions, workshops, and on-going support to researchers interested in developing TILLING in other organisms has been made available through the STP and has served to make the TILLING technique more wide-spread. This group has also developed and made publicly available web-based software programs for PCR primer design and visualization of polymorphisms (Gilchrist and Haughn 2005). Further information about the STP can be found at the following url: http://tilling.fhcrc.org:9366/.

E.2. Zebrafish

Target selected mutagenesis using TILLING is also being done in zebrafish. The Hubrecht Laboratory at The Netherlands Institute for Developmental Biology has generated a library of 4608 ENU-mutagenized F1 animals and has kept a living stock. The DNA from these animals has been screened for mutations in 16 genes using TILLING followed by re-sequencing. This resulted in 255 mutations being identified, 14 of which resulted in a premature stop codon, 7 in a splice donor/acceptor site mutation, and 119 in an amino acid change. They were able to knock out 13 different genes in only a few months time through reverse genetics (Wienholds et al. 2003). Thus far, mutant phenotypes have been characterized for two of the zebrafish genes modified via TILLING - the gene for Dicer1 (Stemple 2004; Wienholds et al. 2003) and the gene for adenomatous polyposis, a tumor suppressor, which through this research was found to have a previously unknown function for signaling in cardiac-valve formation (Hurlstone et al. 2003; Stemple 2004).

F. Limitations and Roadblocks to Completing Reverse Genetics

Completing reverse genetics is not without its pitfalls and not all techniques can be applied to all organisms. In order to be successful, there are several aspects that must be checked. For organisms that do not have efficient transformation systems available techniques such as TILLING that can be applied without transformation may be the only practical choice. In these cases, the rate of mutagenesis is an important factor that can be difficult to determine. The load of mutations must be balanced with the recovery of mutants (Till et al. 2003) – in other words, the genome can't be so riddled with mutations that it is impossible to see a mutant phenotype. Also to be considered is the fertility of the mutagenized organism, especially in the first generation but also in subsequent generations (Perry et al. 2003), both before and after mutagenesis. This is especially true for diploid organisms, because if the sexual machinery is not intact and working properly, then it is impossible to obtain a homozygous mutant. The mutagenized organisms must also be kept alive long enough to screen a mutant population for a specific target. For some organisms, like *Arabidopsis* or *Phytophthora*, this is not a problem as the seeds or cultures are relatively easy to store. It presents a challenge for other organisms, such as zebrafish or rats, because they must be stored and kept alive through the mutant screening stage.

G. Phytophthora

Phytophthora is an oomycete pathogen that is important for many reasons ranging from scientific to economic. The genus has been responsible for a host of diseases and outbreaks with dire consequences, including the Irish potato famine (May and Ristaino 2004; Ristaino 2002) and the more recent Sudden Oak Death (Martin and Tooley 2003;

Rizzo, Garbelotto, and Hansen 2005) epidemic. The economic consequences of damage from *Phytophthora* species is billions of dollars a year in the United States, and much more than that worldwide (Erwin and Ribeiro 1996). Most *Phytophthora* species resemble the true fungi, but are actually more closely related to brown algae and diatoms (Baldauf et al. 2000; Huitema et al. 2004).

Phytophthora diseases can be difficult to diagnose due the similarity of symptoms to other pathogens, causing late recognition and misdiagnoses that often result in heavy losses. Because they are not true fungi they are not affected by most fungicides (Erwin and Ribeiro 1996), so even once identified *Phytophthora* infections are difficult to control. The current effort toward understanding this group of pathogens lies in studying *Phytophtora* at the molecular level, using genome sequence resources to determine their underlying biology (Huitema et al. 2004; Qutob et al. 2000).

P. sojae is a species of *Phytophthora* that is responsible for root and stem rot in soybean, which is considered to be its sole host (Bhat and Schmitthenner 1993; Jackson, Kirpatrick, and Rupe 2004; Leitz et al. 2000; MacGregor et al. 2002; Moy et al. 2004; Tyler 2002; Tyler, Forster, and Coffey 1995; Whissen et al. 1994). *P. sojae* is homothallic, allowing it to self fertilize (Erwin and Ribeiro 1996). This makes it largely inbred and a good background for mutation studies.

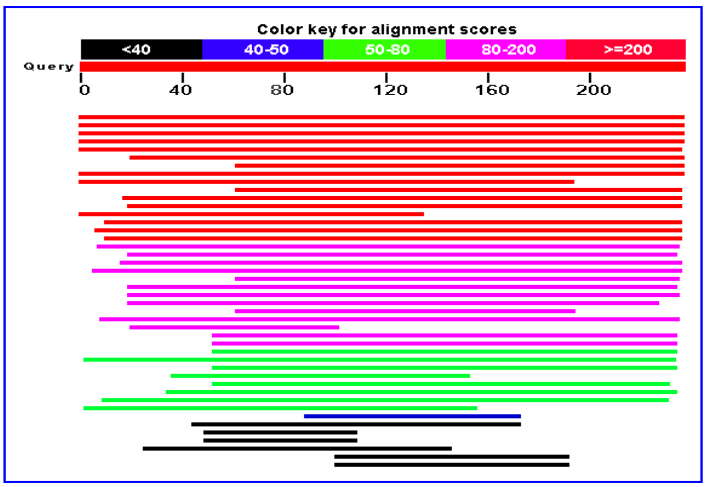
The gene target chosen for this study was *Psoj*NIP (*Phytophthora sojae* Necrosis Inducing Protein). *Psoj*NIP is expressed during the transition from biotrophy to necrotrophy in *P. sojae* infection. The mechanism of necrosis induction is unknown, but studies have indicated that it may occur through manipulation of intrinsic host cell death programs. Evidence also supports that *Psoj*NIP is a secreted protein, an elicitor-toxin

that aids in colonization and accelerates host cell death in the necrotrophic phase of the disease (Qutob, Kamoun, and Gijzen 2002).

*Psoj*NIP is similar in sequence to proteins from other oomycetes, bacteria, and fungi. This can be visualized by performing a BLAST (Basic Local Alignment Search Tool) search at the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/). At the BLAST window of the NCBI website, the position-specific iterated and pattern-hit initiated BLAST (PSI- and PHI-BLAST) was chosen under the protein section. The protein sequence for *Psoj*NIP was entered as input, and the BLAST program then searched through the NCBI protein databases and looked for other proteins containing conserved regions similar to *Psoj*NIP (Figure 2).

H. TILLING Phytophthora

Whole genome sequences for the soybean pathogen *Phytophthora sojae* and the Sudden Oak Death pathogen *P. ramorum* were made public near the end of 2004. Large-scale genomic sequencing projects are underway for the potato late blight pathogen *P. infestans* and the vegetable pathogen *P. capsici*. The compilation of these genomic sequences allows the application of many sophisticated research tools and promises a better understanding of this devastating group of pathogens (Huang et al. 2005; Torto et al. 2003). In an effort to better understand gene function within *Phytophthora* a project to develop a reverse genetic TILLING resource for *Phytophthora* has been initiated. Figure 3 provides an overview of the process as it is being applied to *Phytophthora*. Zoospores present an ideal life stage for mutagenesis as they are uni-nucleate and single mutant individuals can readily be isolated following mutagenesis. The mutation rate for EMS or ENU is not known for *Phytophthora* and lethality is being used as an indicator of



1. necrosis-inducing peptide [Phytophthora sojae]
2. necrosis and ethylene-inducing protein 4-2 [Phytophthora megakarya]
3. necrosis and ethylene-inducing protein 6 [Phytophthora megakarya]
4. necrosis-inducing protein NPP1 [Phytophthora parasitica]
5. necrosis-inducing-like protein [Phytophthora sojae]
6. necrosis-inducing protein NPP1 [Phytophthora infestans]
7. necrosis and ethylene-inducing protein 4 [Phytophthora megakarya]
8. necrosis-inducing-like protein [Phytophthora sojae]
9. necrosis and ethylene-inducing protein 2 [Phytophthora megakarya]
10. necrosis and ethylene-inducing protein 1 [Phytophthora megakarya]
11. 25 kDa protein elicitor [Pythium aphanidermatum]
12. 25 kDa protein elicitor-like protein [Pythium aff. vanterpoolii]
13. necrosis and ethylene-inducing protein 6-2 [Phytophthora megakarya]
14. hypothetical protein BL03562 [Bacillus licheniformis ATCC 14580]
15. necrosis and ethylene inducing protein [Bacillus halodurans C-125]
16. hypothetical protein BLi01595 [Bacillus licheniformis ATCC 14580]
17. hypothetical protein MG08454.4 [Magnaporthe grisea 70-15]
18. hypothetical protein FG06017.1 [Gibberella zeae PH-1]
19. hypothetical protein AN3211.2 [Aspergillus nidulans FGSC A4]
20. NPP1 domain protein, putative [Aspergillus fumigatus Af293]
21. necrosis and ethylene-inducing protein 5 [Phytophthora megakarya]
22. hypothetical protein MG10532.4 [Magnaporthe grisea 70-15]
23. necrosis and ethylene inducing peptide [Fusarium oxysporum f. sp. erythroxyli]
24. NEP-like [Fusarium oxysporum]
25. necrosis and ethylene-inducing protein 3 [Phytophthora megakarya]
26. necrosis and ethylene inducing peptide [Verticillium dahliae]
27. necrosis and ethylene-inducing protein 7 [Phytophthora megakarya]
28. Necrosis inducing [Frankia sp. EAN1pec]
29. putative secreted protein [Streptomyces coelicolor A3(2)]
30. MOSQUITOCIDAL TOXIN PROTEIN [Bacillus thuringiensis serovar israelensis ATCC 35646]
31. hypothetical protein MG00401.4 [Magnaporthe grisea 70-15]
32. putative exported protein [Erwinia carotovora subsp. atroseptica SCRI1043]
33. hypothetical protein FG03394.1 [Gibberella zeae PH-1]
34. hypothetical protein [Vibrio pommerensis]
35. conserved hypothetical protein [Neurospora crassa]
36. hypothetical protein MG02332.4 [Magnaporthe grisea 70-15]
37. hypothetical protein FG07787.1 [Gibberella zeae PH-1]
38. conserved hypothetical protein [Aspergillus fumigatus Af293]
39. hypothetical protein FG11493.1 [Gibberella zeae PH-1]
40. Endoglucanase 2 precursor (Endo-1,4-beta-glucanase 2) (Cellulase 2)
41. endo-beta-1,4-glucanase precursor [Clostridium cellulolyticum]
42. predicted protein [Magnaporthe grisea 70-15]
43. mitochondrial processing peptidase, putative [Cryptococcus neoformans var. neoformans JEC21]
44. hypothetical protein CNBE4620 [Cryptococcus neoformans var. neoformans B-3501A]

Figure 2: Basic Local Alignment Search Tool results aligning *Psoj*NIP with other similar proteins in the National Center for Biotechnology Information database. The colors divide the range of scores into five groups based on similarity.

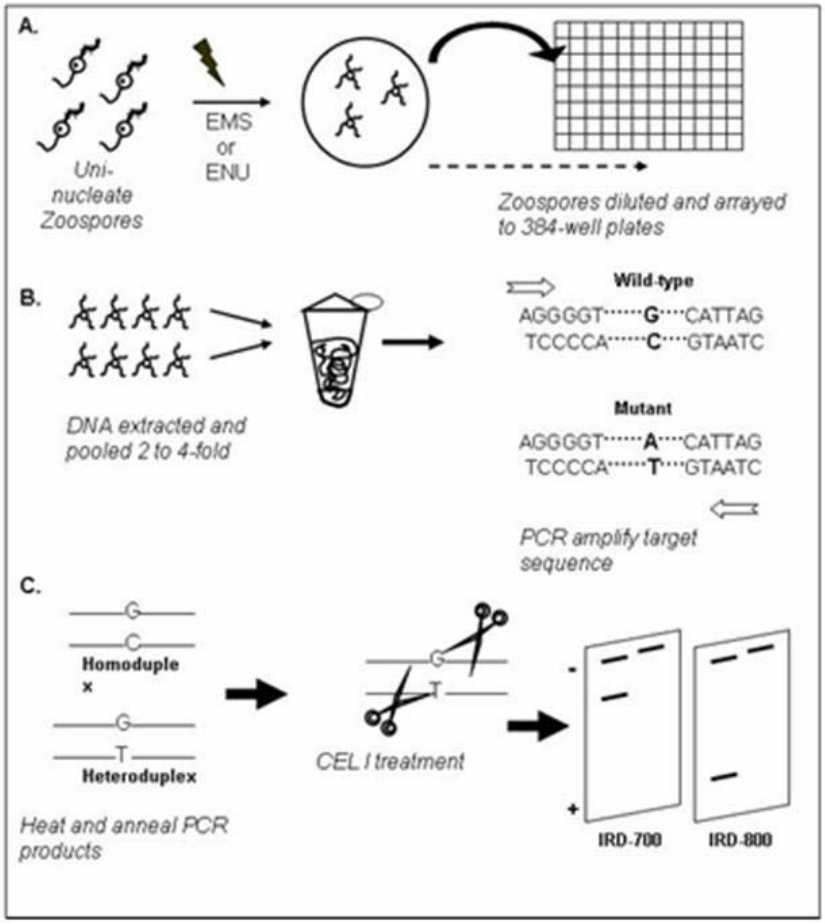


Figure 3: Identification of *Phytophthora* isolates with gene specific point mutations using the TILLING strategy. A, zoospores are treated with EMS or ENU. Single colonies are arrayed in 384-well microtiter plates and grown for long-term storage and DNA extraction. B, genomic DNA is extracted, pooled, and a 1000-bp target amplified with PCR. C, PCR products are heated and re-annealed to form hetero- and homoduplexes, treated with the mismatch endonuclease CEL I, and the resulting fragments resolved on a denaturing polyacrylamide gel. The CEL I enzyme cuts 3' of mismatched DNA and novel fragments are present in pools containing mutant isolates. Positive pools are then analyzed to identify the isolate carrying the mutation.

dose response. Genomic DNA is extracted from the mutant individuals and pooled 2 to 4-fold. The genomic DNA library can then be repeatedly screened. Specific genes are amplified from the pools of genomic DNA and the PCR products are heated up and allowed to cool slowly to form heteroduplexes between wild type and mutant strands ofDNA. The heteroduplexes are treated with the single strand specific endonuclease CEL1 which cuts 3' of single base mismatches producing novel fragments of DNA (Oleykowski et al. 1998; Till, Burtner et al. 2004). CEL1 treated PCR products are then resolved on a polyacrylamide gel and screened for the presence of novel fragments. Pools containing novel fragments are then analyzed to determine exactly which mutant is carrying an induced point mutation and the PCR product from this mutant is sequenced to determine if the induced change is predicted to be silent, missense, or a knockout mutation.

Phytophthora is diploid through most of its life cycle (including the zoospore stage) and all of the induced point mutations exist in the heterozygous state. Once a non-silent point mutation has been identified the mutant isolate is taken through the sexual stage and the sexual progeny are screened to identify individuals homozygous for the mutation under investigation. Homozygous mutants are then tested to determine if there is an altered phenotype.

2. MATERIALS AND METHODS

A. Mutant Library Construction

P. sojae isolate 6497 was used for all experiments. Zoospores were mutagenized at differing rates producing between 15 and 90% lethality. Following mutagenesis the zoospores were diluted and arrayed into 96 or 384-well plates to achieve approximately 1 zoospore per 5 wells. Once the mutant isolates filled the well with mycelium the colonies were transferred to PARP-V8 agar plates and allowed to grow for 5 to 7 days. The isolates were then transferred to both long term storage (2ml tubes with 1ml sterile water and 2 hemp seeds) and to 24-well deepwell plates containing PARP-V8 broth to produce mycelium for DNA extraction.

B. DNA Extraction

Mutagenized *P. sojae* isolates were grown in broth for 7 days and the mycelium harvested into 2 ml deepwell plates containing glass balls (two 3 mm balls per well) and lyophilized for 48 hours. The plates were then sealed and disrupted for a total of 2 minutes to bash the dried mycelium into a fine powder. Genomic DNA was isolated from the samples using the solutions given in Appendix 1 using the protocol outlined in Appendix 2. Concentration of the DNA was estimated by running 3-5µL of each sample of DNA on a 1.5% agarose gel along with lambda DNA of known concentrations. Based on the average concentration of the DNA in each plate, the DNA was then diluted to approximately 0.07 ng/ul for TILLING.

C. Target Selection

Upon consulting the *Phytophthora* research community for genes of interest, *Psoj*NIP (*Phytophthora sojae* Necrosis Inducing Protein) was chosen as a gene target for

this study. *Psoj*NIP is expressed during the transition from biotrophy to necrotrophy in *P. sojae* infection, and is proposed to accelerate host cell death (Qutob, Kamoun, and Gijzen 2002). Primers (Table 1) were optimized using the Codons Optimized to Detect Deleterious Lesions (CODDLE) website (http://www.proweb.org/input/) to amplify ~1,000 nt portions of the gene for *Psoj*NIP (Figure 4) from the mutant genomic DNA.

D. PCR Amplification

A master mix of all PCR reagents except the DNA was made and thoroughly mixed. The primer cocktail (see Appendix 3) is a mixture of both forward and reverse, labeled and unlabeled primers and was pre-made and aliquoted (as fluorescent primers should not be repeatedly thawed and refrozen) and kept in the -80^{o}C freezer. 5µL of master mix was then aliquoted into each well of a 96-well PCR plate and 5µL of mutant genomic DNA was then added to each well (Table 2). The plate was then sealed and the contents quickly mixed and centrifuged, and run on the PCR program described in Figure 5. During this and all subsequent steps, care was taken to shield the reactions from light as the fluorescent primers are light sensitive.

E. CEL Treatment

CEL reaction reagents were mixed together in a master mix (Table 3) and 10µL of the mix was added to each well of the plate containing the PCR reactions. Upon addition of the CEL reagents, the plate was covered with foil tape, vortexed, and centrifuged briefly before being placed into a thermocycler to incubate at 45^{o} C for 15 minutes. After 15 minutes, the CEL reaction was stopped by adding 5µL 75mM EDTA to each well and mixing.

Table 1: Primers targeting *Psoj*NIP

Oligo	Start	Length	Tm	%GC	Sequence
Left Primer	39	24	69.791	50.000	GATTGCCCCGCCTTTTCTTGCTTA
Right Primer	1032	24	69.841	54.167	GCGCGATTAGCGAACGAGATTCAC
Amplified Region		994			

ATCATGACATGCCTACCCACCGAACGGCTCAACGAGGTGATTGCCCCGCCTT
TTCTTGCTTACCTCCTTTGACTCACAGATTCAACAAGCCTTTCCCGCCCAAGA
GGCTGCACGACCTACGTGAATGCTATGCTGCCTCAAAGCTTTGTGCTCTAGAT
CGTGGAGCTCTATCATTTCCCAACTCGCTCCGTCCTGCACACAACAAAACAA
GCTCCTCATCTGACCATGAACCTCCGCCCTGCACTCCTCGCTACGCTGGCTTC
ATTCGCGTACGTGAGCGCCAGCGTTATCAACCACGACCAGGTCGTGCCATTC
ACCCAGCCGACGCCTACGACCGCTCTCCAACAAGCGGCCGTCAAGTACAAGC
CTCAAATCCACATCAGCAACGGCTGCCACCCGTACCCTGCCGTGGACAATAA
CGGCAACACGAGCGGCGGGTTGAATCCTACCGGCAGCGAGAGCGCCGGGTG
CAAGGGCTCCGGCTACGGCACTCAAATCTACGGTCGCGCCGTCAAGTACCAA
GGTGTCTACGCCTTCATGTACTCGTGGTACATGCCCAAAGACGAAACCTTGAC
CGGGCTGGGGCACCGCCACGACTGGGAGGCGTGCGTTGTCTGGGTCGACGAC
ATCGCTGCGTCCAGTCCGAAGATCGTCGCGCTGTCCGCTTCAGCGCACAGCG
GATACAACAAGTACTACCCGCCGAGCTCCTCCTACTTCAGTGGCAACAGCGC
CAAGATCGACTACTCGTCCAGCTACGTGGTCATCAACCACGCGCTGTCGGCC
ACGTCAACTGCGGGCGAGACGCAGCCTCTGATCATGTGGGACCAGCTCACGG
ACGCGGCCCGCAGGGCACTGGAGGACACGGACTTTGGCGACGCCAACGTGC
CGTTTAAGGATGCCAACTTCCAGACCAAGCTCGGCAACGCCTACTACGCTTA
ACGTTGACTCAGGCTTTTAACCTGTCTCGCATACTTGACGGACGGTGCAGCAT
TTGAATGTGACGACTTTGTGAATCTCGTTCGCTAATCGCGCCCTGGTCTTGGC
AGTTTGTAATGCGCCAGGATCCATGGCGTTGAACCTTCCGTTAGCCTCGCCGT
CTCCACGTTTTTGGCGCTTCCGTGACGTCGACTTTGTCGTTC

Figure 4: Gene sequence for *Psoj*NIP. Coding region is in red. Primer sequences are underlined.

Table 2: PCR amplification reaction for TILLING

Reagent	**Volume for 1 reaction**
Sterile Water	3.46
10x PCR buffer	1
5mM dNTPs	0.4
Primer Cocktail	0.04
Taq	0.1
Genomic DNA	5Ml
Total Volume	10Ml

1. 95°C 2 minutes
2. 94°C 20 seconds
3. 73°C 30 seconds
   Decrease by 1°C every cycle
4. 72°C 1 minute
   Ramp to 72°C at 0.5°C/second
5. Cycle to step 2 for 7 more times
6. Incubate at 94°C for 20 seconds
7. Incubate at 65°C for 30 seconds
8. Incubate at 72°C for 1 minute
   Ramp to 72°C at 0.5°C/second
9. Cycle to step 6 for 44 more times
10. Incubate at 72°C for 5 minutes
11. Incubate at 99°C for 10 minutes
12. Incubate at 70°C for 20 seconds
13. Cycle to step 12 for 69 more times
14. Incubate at 4°C forever

Figure 5: PCR amplification of mutant genomic DNA containing *Psoj*NIP. Steps of the PCR program as set on the thermocycler are listed.

Table 3: CEL reaction

Reagent	**Volume for 1 reaction**
Water	7.925 µL
10x CEL buffer	2 µL
CEL I enzyme	0.075 µL
Total	10 µL

F. Sephadex Cleanup

A 96-well deepwell filter plate loaded with Sephadex G-50 Medium (Amersham Biosciences, Uppsala, Sweden) was used to clean up the fluorescently labeled PCR reactions prior to loading them on the polyacrylamide gel. Prior to loading the sample onto the Sephadex, 500µL sterile water was pipetted into each well of a 96-well Whatman Unifilter 800 plate (Whatman Inc., Florham, New Jersey), and the plate was placed on a 1mL deepwell plate and centrifuged at 850xG for 2-3 minutes to clean the column. The water in the deepwell plate was discarded. Sephadex (previously prepared according to the protocol in Appendix 4) was pipetted into each well of the filter plate using a P1000 multichannel with cut tips to load 750µL into each well. The plate was then placed on a 1mL deepwell plate and spun at 850xG for 5 minutes to spin the water through. After spinning, the water from the deepwell plate was discarded and the plate was placed on a PCR plate pre-loaded with 5µL per well of formamide/dye solution. The CEL reactions were then loaded onto the Sephadex columns. Care was taken not to touch the sides of the wells or insert the tips into the sephadex as the sample was slowly applied to the middle of the column. The plate was allowed to sit covered with foil for 5 minutes before centrifuging to bring the sample through the column into the dye plate. The cleaned product in the dye mixture plate was then covered, and incubated at 85 degrees for 45 minutes in a thermocycler.

G. Polyacrylamide Gel Separation of PCR Products

A LI-COR 4300 DNA Analyzer (LI-COR Inc., Lincoln, NE) was used to separate the CEL treated PCR products and is an important tool for detecting the novel fragments produced by single base mismatches. The gel was prepared by the directions according

to the bottle of KB^{PLUS} 6.5% Gel Matrix (LI-COR Biosciences, Lincoln, NE) by adding 150µL of 10%APS (ammonium persulfate) and 15µL TEMED (tetramethylethylenediamine) to 20 mL gel matrix and pouring the solution into the gel apparatus. After being allowed to set (approximately one and one half hours), the gel apparatus is placed in the LICOR and allowed to prerun for 20 minutes. Once the prerun is done, the well is flushed out with buffer and filled with Ficoll dye solution to allow the well to be easily visualized and aid in clearing acrylamide chunks from the well. A 96-well comb loader tray, designed for samples to be loaded with the multichannel pipettor, was loaded with 1ul of sample from each reaction and size ladders labeled with both the 700 and 800 infrared dye. 96-tooth paper combs were used to wick the samples from the comb loader and the paper comb was placed into the well of the gel. After the comb was inserted, the gel was allowed to run for 4 minutes to move the sample into the gel matrix. The comb was removed and the well rinsed before the run was allowed to continue to completion.

Gel images were analyzed using the photo editing program Photoshop (Adobe Systems Inc., USA). Polymorphisms were revealed by the presence of novel fragments in the gel. The LICOR produces two different gel images; one for the spectrum produced by the forward primer which is labeled with a dye that emits signal at 700nm and one for the reverse primer which is labeled with a dye that emits light at 800nm. PCR products harboring a SNP will be cut into two fragments – one labeled with the 700 dye and one labeled 800 dye that together add up to the full length wild type product. This provides a robust means to confirm that novel PCR products shorter than the expected wildtype are the product of CEL cleavage and not spurious products. For example, since the PCR

product is 1000 nt, or 1kb, a novel fragment 600 bases long on the 700 gel image can be confirmed as a SNP with the presence of a 400 base fragment in the 800 gel image in the same lane. This also helps to locate the heterozygous base in the sequencing electropherogram because we know that the SNP is located 600 bases in from one end and 400 from the other. This is illustrated in Figure 6 (see also Figure 3).

H. Sequencing for Single Nucleotide Polymorphism Confirmation

Full length PCR products are amplified and sequenced in both directions for mutants that were found to contain SNP's based on the LICOR gel data. For sequencing, the mutant genomic DNA was PCR amplified with non-fluorescent *Psoj*NIP primers, and then cleaned up using the Qiagen PCR clean-up kit (Qiagen, Valencia, CA). PCR products were submitted to the sequencing facility at the University of Tennessee for sequencing and the trace electropherograms visualized to determine if the SNP previously seen on the gel was in fact present in the genomic DNA.

I. Germination of Oospores

Mutant isolates carrying amino acid changing SNPs were transferred to PARP-V8 agar plates and incubated at room temperature in the dark for 2 to 3 months. Mycelium and oospores were scraped from the top of the plates and thoroughly homogenized using a Tissue Terror (Biocold Scientific, Fenton, MO) and the resulting slurry filtered through a single layer of sterile KimWipe (Kimberly-Clark, Neenah, WI). The filtrate was treated with 0.5mg/ml crude lysing enzymes from *Trichoderma harzianum* (Sigma Aldrich, St. Louis, MO) overnight and the germinated oospores visualized under a light microscope. Germinated oospores were recovered using a suction device constructed from a Pasteur pipette as previously described (Lamour and Hausbeck 2000). Following isolation the

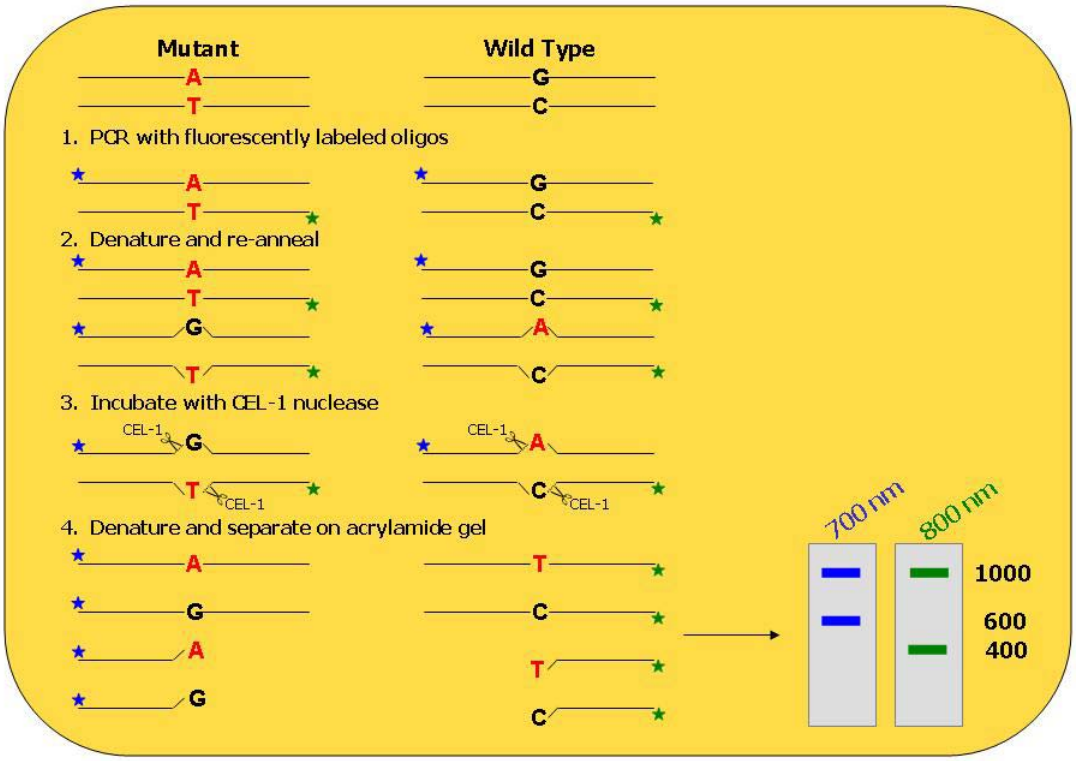


Figure 6: Overview of the mutation detection method.

oospore progeny were analyzed as described for the original mutant isolates.

J. DNA Extraction and Sequencing

DNA was extracted from the mutant progeny in the same manner as from the original mutants (see Appendices 1 and 2). The DNA extracted from the mutant progeny were sequenced to find progeny that are homothallic for the snp. For sequencing, the mutant genomic DNA was PCR amplified with non-fluorescent *Psoj*NIP primers, and then cleaned up using the Qiagen kit as in the earlier step. The sequence data was then screened to determine if the snp previously seen was in fact present in the genomic DNA of the progeny.

3. RESULTS, DISCUSSION, AND CONCLUSIONS

A. Mutant Library

In order to process the large number of mutant isolates needed to build a robust mutant library it was crucial to develop a cost-effective and labor-saving way to extract DNA from *P. sojae.* Over the course of this project the strategies to isolate mutants, grow sufficient mycelium for DNA production, and separate the genomic DNA from other cellular constituents evolved considerably. As of 2005 the mutant DNA production protocol presented here has remained relatively stable and consistently produces high quality, high molecular weight DNA (Figure 7). Currently, there are approximately 3000 mutants and their respective genomic DNA's available for screening.

B. Recovery of Gene Specific Mutants

Primers were optimized to amplify 1,000 nt portions of the gene for *Psoj*NIP (*Phytophthora sojae* Necrosis Inducing Protein) from the mutant genomic DNA. A total of ten SNP's have been observed (Figure 8) and sequenced (Figure 9). Two of these SNP's, in isolates NR108 and NR146, were found to be false positives. Three of the SNP's were found to cause silent mutations – NR562 (Tyrosine to Tyrosine) at position 658, E113 (Tyrosine to Tyrosine) at position 706, and K2302 (Glutamine to Glutamine) at position 265. One SNP, in mutant K2141, was confirmed by sequencing but is 10bp upstream of the coding region. The remaining four SNP's were found to cause an amino acid change. Mutant E16 had an induced change from the wildtype Valine (V) to Glutamic Acid (E) at position 441. This is a drastic change, as V is small, hydrophobic and aliphatic while E is larger, polar, and charged. Mutant E158 has Phenylalanine (F) in place of the wildtype Serine (S) at position 720. The change from F to S is also likely to

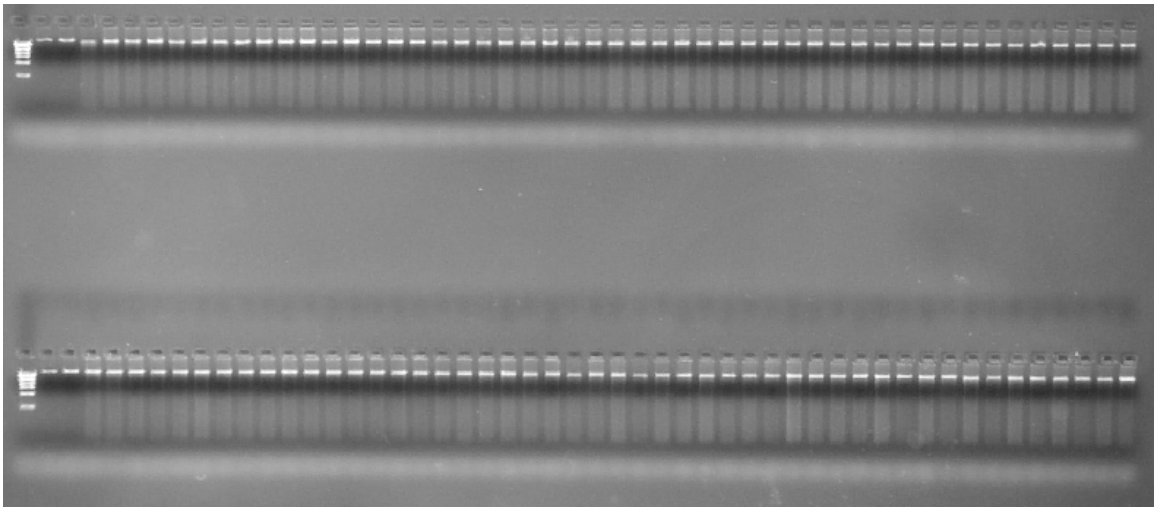


Figure 7: Genomic DNA quantification gel. Agarose gel (1%) with a high mass ladder (L), 10 and 20 ng Lambda DNA (lanes l and 2 respectively), and 3 ul of genomic DNA prepared from approximately 10 mg of lyophilized mycelium from 96 *Phytophthora sojae* isolates (lanes 3 to 48 and 52 to 100). Lyophilization, disruption, and DNA extraction were completed in a 96-well format.

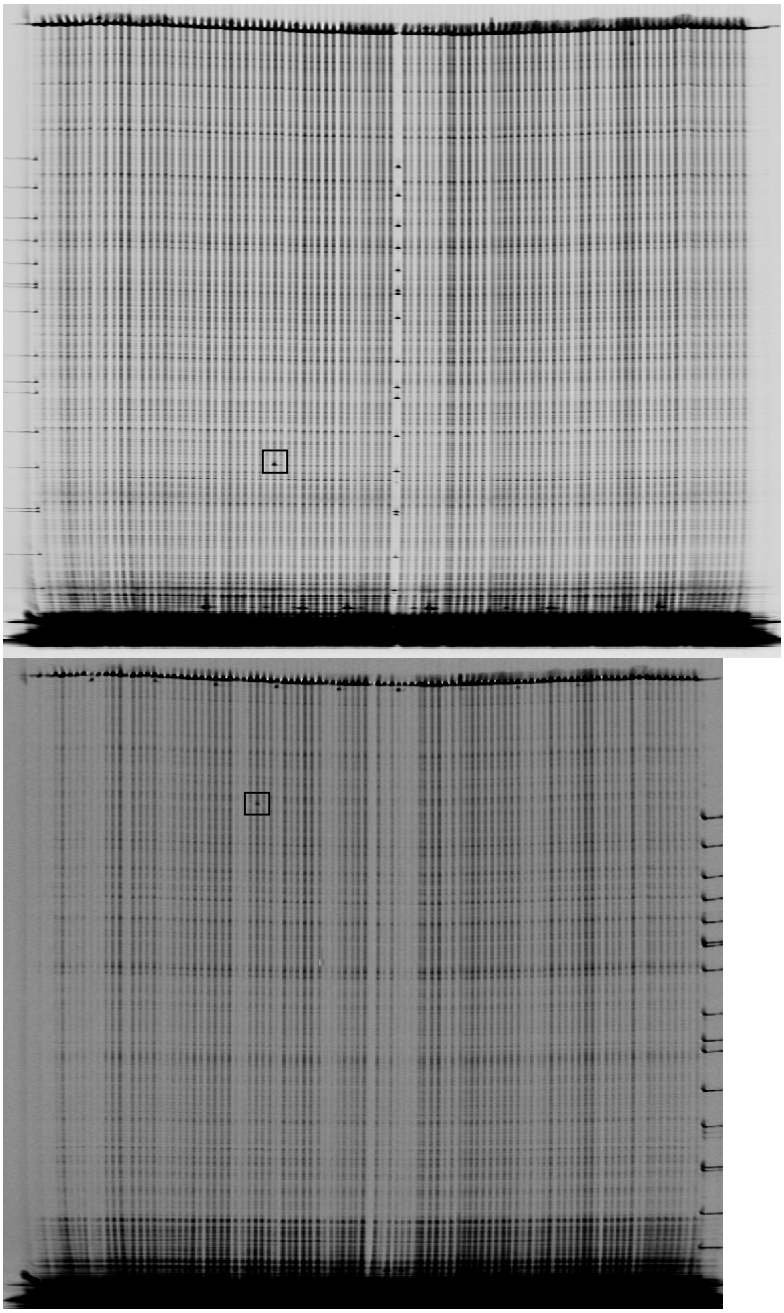


Figure 8: TILLING gel with observed single nucleotide polymorphism (snp). Presence of novel fragments in both gel images in lane 32 adding up to the amplified fragment size (~1000 bp) indicates snp. Top gel is the 700 gel image (fragment size ~260 bp) and bottom gel is the 800 gel image (fragment size ~750 bp).

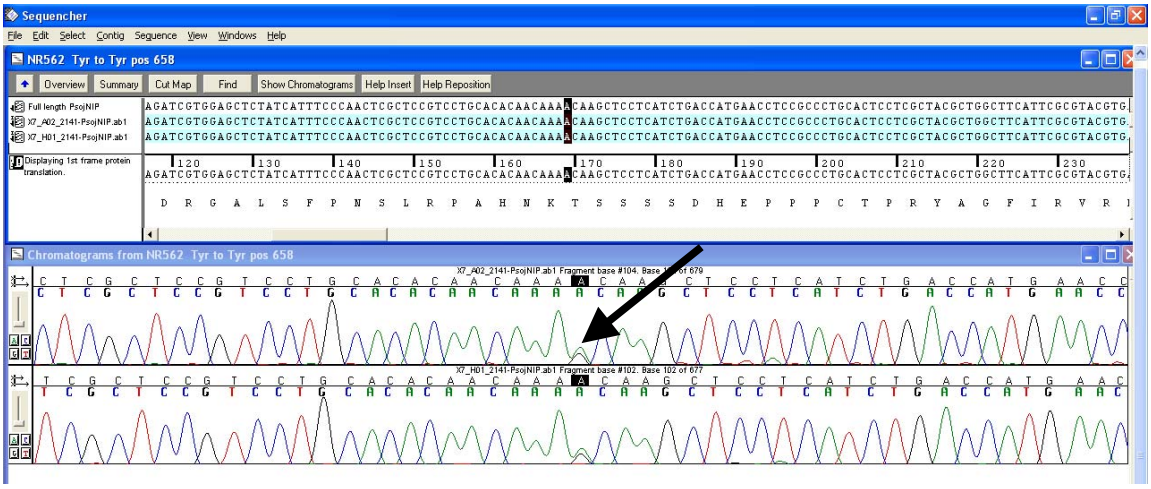


Figure 9: Induced polymorphism confirmed by sequencing. Arrow shows the clear double peak at position 169 which indicates a single nucleotide polymorphism (SNP). In this instance the SNP falls just upstream of the coding sequence and will not impact the gene.

be significant – F is hydrophobic and has an aromatic side chain which, due to its bulkiness, is likely to greatly disrupt protein structure when substituted for the tiny polar sidechain of S. Mutant N37 has an induced change from the wildtype Valine (V) to Isoleucine (I) at position 427. Again, V is small hydrophobic and aliphatic. I is similar to V except that is larger. Although this change is more subtle than the previous two, the size difference could still potentially affect protein folding and structure. Mutant K1857 was a change from wildtype Aspartic Acid (D) to Asparagine (N) at position 557. D is negatively charged, small and polar. N is also small and polar, but it is uncharged. Due to electrostatic forces, this change also has the potential to alter protein folding and function.

From the CODDLE website (http://www.proweb.org/input/), predictions can be made based on the mutation method as to what types of mutations will result in your sequence of interest. When the *Psoj*NIP sequence was entered, the following mutations were predicted (Table 4). CODDLE also calculated that 57.3% of the changes would be

Table 4: CODDLE mutation predictions

Mutation Type	**Percentage of total mutations**
A ↔ T Transversions	44.0%
A:T → G:C Transitions	38.0%
G:C → A:T Transitions	8.0%
A:T → C:G Transversions	5.0%
G ↔ C Transversions	3.0%
G:C → T:A Transversions	2.0%

nonsilent, with 1.8% of these polymorphisms being truncation (nonsense) changes and 55.5% being missense changes. These numbers correlate with the experimental data described above

C. Oospore Progeny

The next step was to recover sexual progeny from the mutants that are homothallic for allelic and knockout mutations. *Phytophthora sojae* is self-fertile and makes sexual oospores in single culture. Unfortunately, the wildtype copy of isolate P6497 that we obtained from another *Phytophthora* research group at the start of this project does not produce normal oospores. This has made it very difficult to recover sexual progeny from the mutants harboring amino acid changing mutations. We have since procured another copy of P6497 that produces normal oospores from the long term *Phytophthora* culture collection at the University of California, Riverside. Attempts are being made to salvage the mutants isolated from the defective *P. sojae* isolates (E16 and E158) isolated during the course of this work. Due to the very low number of normal oospores produced by the mutant isolates described here and the need for extended periods of dormancy (2 to 3 months) for oospore germination, we have only been able to analyze six sexual progeny from the E16 mutant parent. Sequencing of the *Psoj*NIP gene for these six isolates did not reveal any carrying the homozygous mutant allele.

D. Conclusions

TILLING was successfully adapted to *P. sojae* and *Psoj*NIP specific mutants were recovered. Further research using a *P. sojae* strain that is more efficient at producing oospores will be required to ascertain the heritability of the mutations and the effects on pathogenicity of the mutants.

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APPENDICES

APPENDIX I: SOLUTIONS FOR DNA EXTRACTION

AP1 = Lysis Buffer

100 mM Tris (pH 8.0)	100 ml 1M Tris stock
50 mM EDTA	100 ml 0.5M EDTA stock
500 mM NaCl	29.2 g NaCl
1.33% SDS	13.5 g SDS

Start with about 500 ml MilliQ water in a 1 liter beaker with a stir bar. Add the liquid stock solutions first. Add the NaCl and SDS (wear a face mask when weighing SDS). Stir until in solution and bring final volume to 1 liter. Store in a 1 liter glass bottle and do not autoclave.

AP2 = Potassium Acetate (pH 8.0)

Make a 5M solution of KOAc. Weigh out 245.4 g potassium acetate and add to 200 ml of MilliQ water stirring in a beaker gradually. Add water to bring volume to 500 ml. Using the pH meter, add glacial acetic acid to bring pH to 8.0. Filter sterilize.

AP3 = Guanidine Hydrochloride and Alcohol

Make a 5 M stock of Guanidine Hydrochloride (GH). Dissolve 238.8 g of GH in MilliQ Water (start with small amount, 200 ml). Bring final volume to 500 ml. Filter sterilize. Use 125 ml of 5M GH to add to 250 ml 95% EtOH. Keep stock solution of 5M GH for use later.

AW = Tris, EDTA, NaCl stock (AW salt stock).

33 mM Tris (pH 8.0)	16.5 ml 1 M Tris
3.3 mM EDTA	3.3 ml EDTA
165 mM NaCl	4.82 g NaCl

Dissolve in 200 ml MilliQ water and bring final volume to 500 ml. Filter sterilize and keep as salt stock for AW.
Mix 300 ml of the salt stock with 700 ml EtOH to make home-made AW.

AE = 10 mM Tris (pH 8.0)

To make 1 liter, add 10 ml 1 M Tris to 990 ml MilliQ water. Autoclave and aliquot into sterile 15 ml or 50 ml tubes in the sterile hood.

APPENDIX 2: PLATE DNA EXTRACTION PROTOCOL

1. □ There are pre-made bottles of AP1. Use one bottle per 2 plate extraction. Heat the bottle in the 65º oven until the chunks are gone. Heat tube of Fighter F at same time.
2. □ While heating - bash the samples for 30 seconds on each side on a setting of 30. Make sure to rotate the plates and do each side at this setting. After the first bash – check to make sure there is powder in each well. **If no powder – then there is probably no beads so you'll need to add 2-4 beads to the well.** Spin the plate for five minutes and carefully remove cap and put it in the sink.
3. □ Add 800µl of Fighter F and 200µl of RNase to the preheated AP1. Don't fret if the fighter F is finicky – just get as much as you can in there. Shake the tubes vigorously.
4. □ Add 500µl of the AP1 mix. Put a cap on, and gently shake the plates until all the chunks of mycelium are in solution.
5. □ Incubate the plate in the 65º oven for 30 minutes. Make sure the cap is sealed (it gets loose during the incubation). Spin for 1 minute at max speed and take the cap off (never reuse caps!).
6. □ Add 200ul AP2 Buffer using the 8-channel pipette (there are pre-made tubes with enough for 2 plates) to the plates, add a new cap, and shake the plate vigorously to mix the solutions for 15 seconds. Put in the -20C freezer for 20 minutes or leave in the -20C overnight.

AFTER FREEZING:

7. □ Spin plates for 10 minutes at max speed. If you left the plates in the -20° freezer overnight, let them stand at RT for a few minutes before spinning the plates. If you spin them frozen, nothing will happen!
8. □ While the plates are spinning add 800µl of AP3 Solution (there are pre-made tubes of AP3 for 2 plates) to properly labeled 2ml plates that will hold the AP3+AP2 mixture. Use the 8 channel pipette.
9. □ Transfer 500µl of the supernatant from the spun plate to the correctly oriented/labeled deepwell plate from step 8. The goal is to get only supernatant. There is a mark on the Apricot in rm 403 – use this. If you look very closely you can see where the tips of the multichannel are in the plate. Try to get them just above the precipitated sludge. Have Kurt help you do this the first time.
10. □ Put a new cap on the plate, shake the plate vigorously for 15 seconds, and spin the plate for 1 minute at 5220 RPM.

11. □ Put a properly labeled/oriented glass filter plate on a clean 2 ml deepwell plate. Be sure to label the 2ml plate properly.
12. □ Transfer 1300µl (two 650 µl aliquots using the 8 channel) to the glass filter plate.
13. □ Spin at 5220 RPMs for 5 minutes. Discard flow through.

14. □ Add 800µl of Buffer AW. There is premade AW in tubes for 2 plates. Cover with a rayon breathable tape. Spin for 5 minutes. Discard flow through.
15. □ Add 400µl 95% ethanol. Cover with a rayon breathable tape. Spin for 5 minutes. Discard flow through.
16. □ Put AE buffer (to preheat) into the 65º oven and ALSO put the spin plate (alone, without the 2 ml deepwell plate and without the rayon tape) into the 65º oven to dry for 15 minutes. There is prepared AE in tubes for 2 plates.
17. □ Place the dried glass fiber plate onto a new (never used) properly labeled and oriented 1ml deepwell plate.
18. □ Put 200µl of preheated AE buffer into the plate. Let it sit 30 minutes at room temperature.
19. □ Spin for 2 minutes at 5220 RPM.
20. □ Run 3µl DNA from Rows B and G on a 1% agarose gel. (3 ul DNA + 3 ul Tris + 2 ul dye).
21. □ Seal the plate with the thick tinfoil tape (expensive tape). Store the labeled DNA plate in the refrigerator and record on the log that the DNA has been extracted.

APPENDIX 3: PRIMER COCTAIL RECIPE

To make primer cocktail, mix following in a tube and aliquot into small volumes (enough for 2 gels; about 10µl per tube works well)

All primers at 100 µm concentration

24 µl L700 blue primer
16 µl L unlabeled primer
32 µl R800 green primer
8 µl R unlabeled primer

APPENDIX 4: SEPHADEX PREPARATION

Weigh out 11 grams of Sephadex and mix in 160 mL MilliQ water in glass bottle

Autoclave 15 minutes.

This makes approximately enough for 2 plates.

VITA

Melinda Tierney was born Melinda Beth Lawrence on December 29, 1980 in Rolla, MO. She was raised in Joplin, MO and attended Royal Heights Elementary School, North Middle School, and Joplin Junior High. She graduated from Joplin High School in 1999. From there, she went to the University of Missouri-Rolla and met and married John Tierney. She graduated from UMR in December of 2003 with a B.S. in Biological Sciences and minors in Chemistry and Psychology. Melinda was then accepted to the Genome Science and Technology program with the University of Tennessee and Oak Ridge National Lab and moved to Knoxville, TN to pursue graduate work. In December 2005, Melinda earned a Master of Science degree in Life Sciences.