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Cloning and characterization of a soybean SNF2 yeast homolog

by

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has met the dissertation requirements of Iowa State University

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For the Major Program

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For the Graduate College

DEDICATION

To Ms Shoufeng Zhong who has been a mother to me

for her love and caring

To my mentor, Professor Alan G. Atherly,

for his generosity and guidance

TABLE OF CONTENTS

ABSTRACT

It is of great economic and scientific interest to understand plant disease resistance genes at the molecular level. *Phytophthora sojae*, a fungal pathogen of soybean is of particular interest since the root and stem rot resulting from its infection causes great economic loss. It is the goal of this project to clone *Phytophthora* resistance genes from soybean. This was done by designing primers from the conserved regions of known plant disease resistance genes using a modified differential display for near isogenic lines; L85-3044 a resistant line and Williams, a susceptible line. Several polymorphic bands were identified, including an unique 0.7 kb band. This DNA fragment was cloned and used to screen libraries. To accomplish this, genomic and cDNA libraries of L85-3044 were constructed. Several interested clones have been identified. One of the positive cDNA clones was identified as gmSNF2 that was a yeast SNF2 gene homolog. The putative gmSNF2 protein has, a leucine zipper motif, a ATP binding motif and a leucine content of 11%. It also has a 39.0% similarity to six known plant disease resistance genes. Genetic complementation test of yeast SNF2 mutant indicate that soybean gmSNF2 gene has SNF2 function in yeast. The SNF2 gene of yeast is a member of a large group of related proteins that exhibit a broad range of biological functions, such as gene-specific transcriptional activation, and transcriptional repression. To determine if the gmSNF2 would

restore pathogen resistance to susceptible lines, the putative resistance genes were introduced into the roots of soybean by *Agrobacterium rhizogenes* mediated transformation. A protocol for screening *Phytophthora sojae* resistance in transgenic roots was also developed based on color assay and thin layer chromatography. The analysis of transgenic susceptible varieties Williams and Hark showed that gmSNF2 could produce significant resistance-reactions. We thus hypothesize that the gmSNF2 gene may sense the free sugar molecular released from fungal wall by soybean glucanases and initiate a sugar mediate signal transduction pathway for *Phytophthora sojae* resistance.

CHAPTER I. GENERAL INTRODUCTION

Mapping, cloning and characterization of plant disease resistance genes is important in several respects. First, it is helpful to plant breeders in combating plant diseases through molecular marker assisted backcrossing programs, and secondly molecular geneticists can use this information to gain an understanding of the mechanism of interaction between plant pathogens and host plants. Through both of these approaches, optimism exists for eventually controlling many plant diseases that frequent cultivated crops. In this chapter, I will briefly review information relating to the cloning of various plant disease resistance genes as well as their bacterial counterparts. In addition, I will discuss data and information connected to *Agrobacterium* mediated transformation of soybean. In this thesis, *Agrobacterium* mediated transformation of soybean was used to verify the putative *Phytophthora* resistance gene candidates in a cloning project.

Plant disease resistance genes

In 1947, based on the studies of interaction of flax with the rust fungus, Flor proposed the gene-for-gene hypothesis. This hypothesis states that a dominant gene confers resistance to a specific pathogen race, but only if the pathogen expresses the corresponding avirulence gene (Flor 1971). These two complementary genes, one in the pathogen and the other in the plant, can now be interpreted to mean that the pathogen produces an elicitor (a protein or other molecule attached to that protein) which recognizes a protein produced by the plant. This interaction triggers a cascade of defense reactions for the plant (Callow 1977; Keen 1993; Mehdy 1994). This hypothesis has fascinated plant molecular biologist for decades and has recently culminated in the cloning of at least 12 plant resistance genes.

There are two general types of plant disease resistance. One general mechanism is horizontal resistance, and the other is pathogen race specific. Horizontal resistance, which is generally polygenic, is difficult to characterize at the molecular level. This type of resistance includes heavy physical barriers for the entrance of pathogens into plant leaves and stems, and the production of antimicrobial substances such as phytoalexins, toxins and enzymes (Keen 1992; Mehdy 1994; Bent 1996). The subject of this thesis, resistance to *Phytophthora* root-rot in soybeans, belongs to the race-specific or vertical resistance.

Phytophthora sojae

Phytophthora root and stem rot of soybean is one of the most destructive fungal diseases in the soybean-production areas of the USA and other countries. and has resulted in an estimated $188 million loss per year in 1988-1991 (Doupnik 1993) in the USA alone. Six loci have been identified which contain alleles possessing race-specific resistance to *Phytophthora*. Soybean variety Williams contains the recessive alleles conferring susceptibility at all six resistance loci. A near-isogenic line (NIL) L85 contains resistance alleles at these loci (Diers et al. 1992). The near isogenic lines were obtained by backcrossing six times from a resistant recurrent parent.

A simple 3:1 inheritance ratio for resistance has been reported for *Phytophthora sojae* race 1, 2, 3, 4 and 5 in various soybean varieties (Diers et al. 1992). The race-specific resistance generally has a high level of resistance, but only against a particular race of the pathogen, and it is generally a dominant single gene which determines resistance (Keen 1992, 1993).

The elicitor-receptor hypothesis states that pathogen avirulence genes specifically produce unique signal molecules, called elicitors, that are specifically recognized by only the plant genotypes carrying the complementary disease

resistance genes (Callow 1977: Keen and Dawson 1992). The first cloned fungal avirulence gene, avr9, from *Cladosporium fulvum* encodes a peptide elicitor for tomato varieties carrying the Cf9 resistance gene (Keen et al. 1990). Since the cloning of this gene from *C. fulvum*, other pathogenic avirulence genes have been cloned and are listed in Table 1. However, it was found that not all avirulence genes act as direct elicitors. An example is the *avrD* gene from *Pseuodomonas syringae pv.* tomato, which is involved in the production of small elicitor molecules (gama-lactones) that trigger resistance in tomato varieties carrying Cf9 genes (Keen et al. 1990). It was also reported that elicitins, which are a family of small proteins secreted by species of *Phytophthora,* were found to be negatively correlated with their pathogenicity on tobacco plants (Yu 1995). In *Phytophthora sojae*, there are at least two class of elicitor molecules. One class belongs to the oligoglucans (Keen and Dawson 1992; Ebel et al. 1993) and the other class are oligopeptides (Nurnberger et al. 1995). The soybean β-glucan elicitor binding protein has been cloned (Umemoto et al. 1997), but it does not have any common conserved motifs of plant disease resistance gene as discussed in the next section. The oligopeptides elicitor receptor protein has also been cloned and reported as a specific MAP kinase from parsley (Ligterink et al. 1997).

Molecular characterization of plant disease resistance genes

A discussion of the molecular structure of plant resistance genes has been the subject of several reviews (Lamb 1994; Staskawicz et al. 1995; Dangl 1995; Jones 1996; Kunkel 1996; Boyes et al. 1996; Bent 1996). Table 1 summarizes information on the 12 cloned plant disease resistance genes. These cloned resistance genes can be classified into five groups according to their molecular structure and locations within the cell. Figure 1 shows a comparison of the

Table 1. Disease resistance genes in plants and avirulence genes

Group	Gene	Sources	Pathogen (aviruience gene)	Major Motifs	Cloning Methods	References
1	Rps2	*A. thaliana* (2n=10)	Bacaterial *P. syringae* (avrRpt2)	P-loops, GLPLAL LLLLDD, LRRs, LZ, potential-N-glycosylation sites	Positional cloning	Mindrinos, et al., 1994; Bent,etal., 1994
1	N	Tobaco (2n=24)	Mosaic virus	P-loops, GLPLAL LIVLDD, LRRs potential-N-glycosylation sites	Transposon tagging	Whitham, et al., 1994
1	L6	Flax (2n=30)	Rust Fungus *C. carbonum* Race 1	signal peptide P-loop, GLPLAL LVVLDD, LRRs, LZ	Transposon tagging	Lawrence, et al., 1995
1	Prf	Tomato *P.s. tomato* (avr*Pto*)	Bacterial	P-loop, LRRS, LZ GLPLAI, IVVLDDV	Positional cloning	Salmeron et al., 1997
1	RPM1	Tomato	Bacterial *P. s.pv maculicola* (avrPpm1 & avrB)	P-loop, LRR, LZ ILVDDV	Positional cloning	Grant et al., 1995
2	Cf-9	Tomato	Fungus *C. fulvum* (Avr9)	LRR N-glycosylation sites Transmembrane dormain	Transposon tagging	Jones, et al., 1994;
2	Cf-2	Tomato	Fungus *C. fulvum* (Avr2)	LRR N-glycosylation sites Transmembrane dormain.	Positional cloning	Dixon, et al., 1996
2	Xa21	Rice (2n=24)	Fungus *Xanthomonas oryza*	LRR, Kinase motifs Transmembrane dormain	Positional cloning	Song, et al., 1995
3	Pto	Tomato	Bacterial *P. syringae* (avrPto)	DLKPEN, G(T/S)XX(Y/F)XAPE	Positional cloning	Martin, et al., 1993
4	NPR1	*A. thaliana*	Bacterial Psm ES4326 Fungus, *P. parasitica*	ankyrin repeats	Positional cloning	Cao, et al., 1997
4	Mlo	Barley (2n=14)	Fungus *Erysiphe graminis f.sp.hordei*	6 helices motifs, 2 casein kinase II sites	Positional cloning	Büschges et al., 1997
5	HM1	Maize	Fungus (2n=20) *Cochliobolus carbonum*	None	Transposon tagging	Hohal, and Briggs, 1992

LZ=lucine zipper; LRR =lucine reach repeats. The bacterial disease resistance gene is indicated when known.

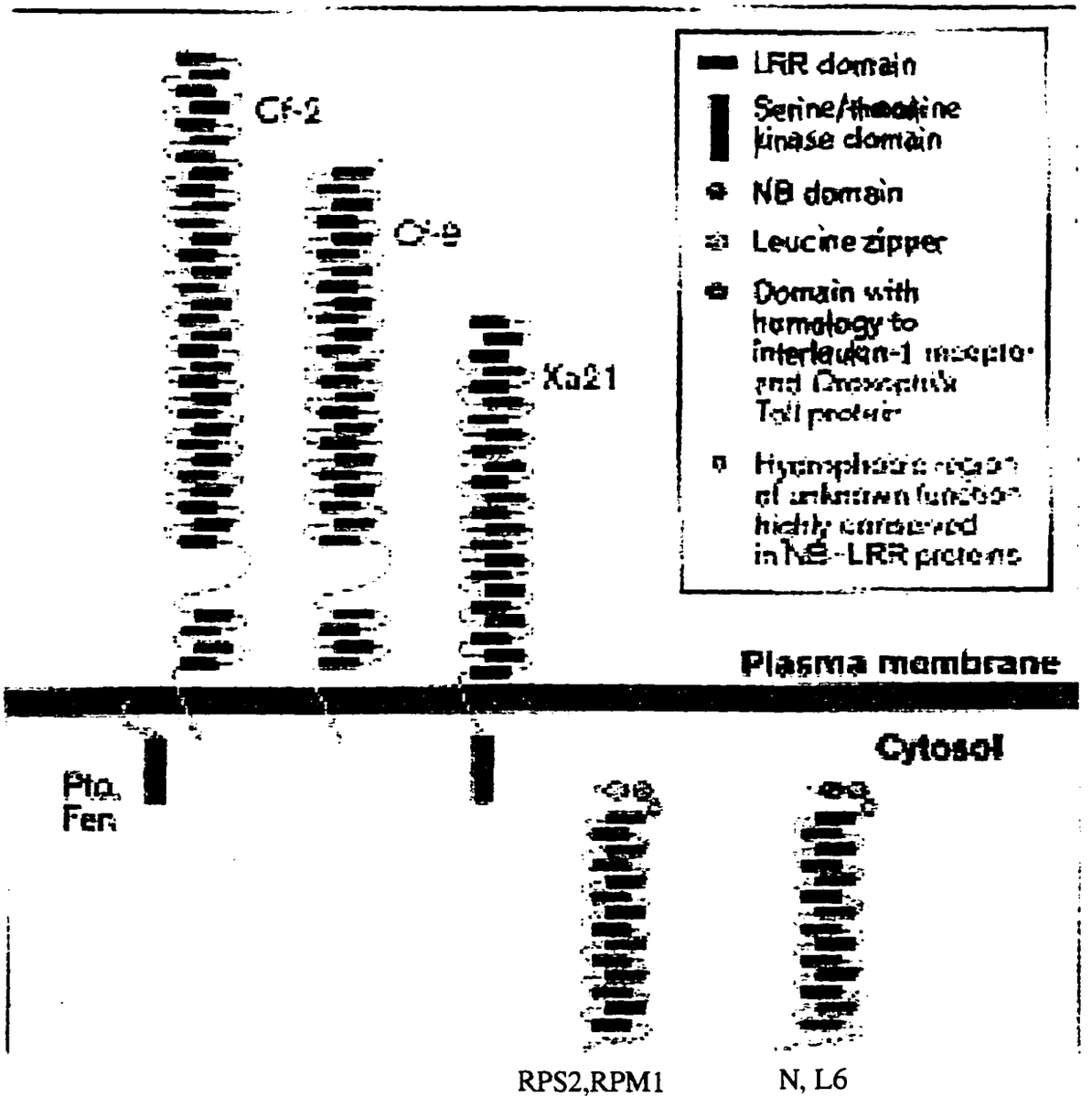

Fig.1 A comparision of putative protein sequences from some of known plant resistant genes (Adapted from Boyes et al., 1996)

protein sequences of plant disease resistance genes. The derived amino acid sequence of the first group of plant disease resistance gene products are located in the cytosol and contain a leucine-rich repeat (LRR) domain in the C-terminal portion of the protein (Moffat 1994). All proteins containing LRR motifs are thought to be involved in protein-protein interactions or binding of peptide ligands (Kobe and Deisenhofer 1994). The resistance genes in this group also have three common conserved motifs. The first common conserved motif is called the P loop and is in the N terminal portion of the protein. The P-loop is an amino acid sequence that binds specifically to phosphate nucleotides in energy reactions (Saraste et al. 1990). The second conserved motif is a kinase-2 motif or VVLDD. The third conserved motif is GLPLA, which is in a membrane-integrated region and is located at the approximate center of the protein. Two other conserved domains, kinase 3a and conserved domain 3, (Grant et al. 1995) have similar domains within their groups, but are less conserved than the P-loop, kinase-2 and GLPLA motifs.

The first cloned resistance gene of group 1 was RPS2 from *Arabidopsis thaliana* that confers resistance to the bacterial pathogen *P. syringae* carrying the avirulence gene *rpt2* (Bent et al. 1994; Mindrinos et al. 1994). Other resistance genes in group 1 are tobacco mosaic virus resistance gene N (Whitham et al. 1994), L6 from the flax rust resistance gene (Lawrence et al. 1995), RPM1 gene from *Arabidopsis thaliana* that confers resistance to bacterial pathogen *P. syringae* carrying the avirulence gene *rpm1* (Grant et al. 1995; Leister et al. 1996), and the Prf gene from tomato which confers resistance to *P. syringae tomato*, carrying the avirulence gene *pto* (Salmeron et al. 1996). However, RPS2, Prf and RPM1 in this group have a unique putative leucine zipper motif. The leucine zipper motif is found in many DNA-binding proteins where two alpha

helices from separate proteins are joined together in a coiled-coil, forming a protein dimer (O'Shea et al. 1989).

The protein sequences of the second group of resistance genes also contain LRRs but proteins from this group may form an extracellular rod that may interact with interacting elicitors. Cf-9 and its related gene Cf-2, confer resistance to the fungus *Cladosporium fulvum* in tomato fall into this class (Dixon et al. 1996; Jones et al. 1994). Cf-9 and Cf-2 are unique as they possess a transmembrane motifs. Another plant disease resistance gene, Xa21 from rice, confers resistance to *Xanthomonas oryza pv. oriza* race 6 and is unique due to the presence of an intracellular kinase-like domain in its protein product (Song et al. 1995).

A third group of plant disease resistance genes possess an adjacent receptor kinase-like gene cluster (Hanks 1987), such as the Pto gene from tomato, which confers resistance to *Pseudomonas syringae pv. tomato.* The bacterial counterpart expresses the *pto* avirulence gene product (Martin et al. 1993). Analysis of the amino acid sequence of the Pto gene from tomato reveals similarities to serine-threonine protein kinases. This suggested a role for Pto in a signal transduction pathway. The model for Pto perception of the *avrpto* signal and its induction of a hyper susceptible response has been recently reviewed (Lamb 1994; Martin et al. 1994; Bent 1996). The phosphorylation activity of Pto with a similar strain serine/threonine kinase gene has also been reported (Martin et al. 1994; Zhou et al. 1995). This was the first report that proved that a plant disease resistance gene, Pto, could specifically phosphorylate its possible downstream gene, Pto1, in a signal transduction pathway, but not vice versa.

The fourth group of genes are those that have unique protein motifs such as Mlo from barley, which confers a broad spectrum resistance to the fungal pathogen *Erysiphe graminis f.sp.hordei* (Buschges et al. 1997). Mlo has six

membrane-spanning helices and two of casein kinase II sites. Gene NPR1 from *Arabidopsis thaliana* controls systemic acquired resistance that contains ankyrin repeats (Cao et al. 1997). The ankyrin repeats consensus motif was found in various groups of proteins involved in cell structure, transcriptional regulation, cell differentiation, and enzymatic and toxic activities (Bork 1993).

The last group of disease resistance genes includes Hm1, that was the first plant resistance gene cloned. Hm1 encodes a reductase that confers resistance against specific strains of the fungal pathogen *Cochliobolus carbonum* by degrading the fungal HC toxin. It does not have any known motifs and it is the only gene that does not strictly conform to gene for gene hypothesis (Jones 1996). But, it has provided an important mechanism for providing plant disease resistance (Bent 1996). The Hm1 gene from maize was cloned by a transposon tagging strategy (Johal and Briggs 1992). This involved using the transposon Mu1 to interrupt the target gene in F1 plants and then using the interrupted sequence as a probe to identify the flanking region of the target gene from a genomic library.

There have been two hypotheses with respect to the existence of similar molecular structural motifs in plant disease resistance genes. One hypothesis suggests that there is a grand "unified" response pathway in plant disease resistance (Briggs 1995). However, this hypothesis has been questioned because different responses are employed for different pathogens, and possibly for different strains of the same pathogen (Boyes et al. 1996). The second hypothesis suggests that plant disease resistance may have a common molecular mechanism in the signal transduction pathway (reviewed by Jones 1994; Staskawicz et al. 1995; Dangl 1995; Kunkel 1996). It is not yet known what role consensus motifs have in the common molecular mechanism for signal transduction pathway of plant disease resistance. On the other hand, it may be

possible to clone additional disease resistance genes using PCR, designing primers from the common conserved motif sequences in these genes. This procedure, in combination with a modified differential display, has been used in this thesis.

Strategies for cloning plant disease resistance genes from soybean

All the plant disease resistance genes listed in Table 1 have been cloned by either transposon tagging or map based positional cloning. Each approach has its advantages and disadvantages. Map based cloning is dependent on the availability of closely linked markers flanking the desired resistance gene (Tanksley et al. 1995), and this situation is sometimes tedious to obtain. Transposon tagging is dependent upon a transposable element system in the plant in question. Some plants, such as soybean, do not have a characterized transposon tagging system. From the studies of Diers et al. (1992), a partial RFLP map was begun in soybean. They identified the polymorphic RFLP markers pT5 and pa-586 that map close to the Rps5 locus of soybean, but used a small population of F2 progeny (56) in their mapping analysis (Diers et al. 1992). Thus, the error associated with the map position could be significant (Tanksley et al. 1995). Their data also show that both pT5 and pa-586 are linked to Rps4. These results suggest that the two loci may be in linked clusters.

The soybean (2n = 40) is regarded as a stable tetraploid with diploidized genomes (Hymowitz and Singh 1987). It has more than 60% of repeat sequences scattered among single copy regions (Goldberg 1978) and its pachytene chromosomes indicate that over 35% of the genome consists of heterochromatin. The short arms of six of the 20 bivalents are completely heterochromatic (Singh et al. 1988). It was hypothesized that genes are preferred sites for meiotic recombination (Werner et al. 1992; Civardi et al. 1994). Meiosis induced double

strain break sites for the recombinations were determined by adjacent chromatin structure (Wu and Lichten 1994, 1995). Plant disease resistance genes frequently occur in tightly linked clusters or as true alleles (Keen 1992). Thus, it is likely that map based cloning of genes from soybeans would be more difficult than in other simple diploid crop species, even if closely linked markers have been identified using bulked segregate or pooled populations (Michelmore et al. 1991; Churchill et al. 1993; Tanksley et al. 1995). Since more than two copies of a gene may be present in the genome, recombination between the polymorphic linked markers and a target gene may be inhibited by its location around heterochromatin, thus linkage information may be biased. Thus it may require a considerable amount of labor to walk down the soybean genome.

An alternative approach is to design primers from common conserved motifs in plant disease resistance genes and utilize the polymerase chain reaction (PCR) to amplify putative genes. Using this approach, several groups have reported finding disease resistance gene analogs in soybean (Kanazin et al. 1996; Yu et al. 1996). The disadvantage of this method is that it is only possible to clone disease resistance genes that have the similar molecular motifs.

Another procedure that has been developed in the past eight years is useful in identifying rare mRNAs synthesized in one strain but not another. This procedure, known as differential display, utilizes reverse PCR(RTPCR) to generate arbitrary primed DNA fragments from two near isogenic lines or a mutant vs a wild type. These DNA fragments are then resolved on sequence gel or agarose (Liang and Pardee 1989; Yoshida et al. 1994). The two RTPCR products are then compared and any mRNAs diffrentially expressed are identified and studied. To increase the efficiency for expression analysis of multigene families, Fisher et al. (1995) proposed the restriction fragment length polymorphism-coupled domain directed differential display.

As a result, I have used this approach and designed primers using common conserved motifs, specifically P-loop domains, from plant disease resistance genes. The main results from these studies are presented in chapter 2.

Agrobacterium mediated transformation of soybean

If a putative disease resistance gene is cloned, it is necessary to reintroduce the gene back into a susceptible line for a final proof that the gene is involved in disease resistance. Unfortunately, no easy procedure is available for transformation of soybeans. One approach is to utilize hairy roots generated by *Agrobacterium rhizogenes*, where the putative disease resistance gene has been introduced into the roots only. It is possible to obtain expression efficiency as high as a 37% for *Agrobacterium rhizogenes* mediated hairy roots infection on some genotypes of soybean (Savka et al. 1990). But there is no efficient *Agrobacterium* mediated transformation procedure useful on agronomically important soybean varieties. Thus, one objective of these studies was to improve the *Agrobacterium rhizogenes* mediated transformation efficiency and test cloned putative disease resistance candidate genes. Another problem in the transformation of genes into soybeans is related to highly variable levels of gene expression after transformation. If a gene is transformed into a soybean line, and found not to be effective in reversing disease resistance, it would not be obvious whether it was due to a low level of expression (or no expression) or lack of participation in the disease resistance process. We asked the question whether the low *Agrobacterium* mediated transformation efficiency and varying levels of transgenic gene expression in soybean were due to the high percentage of methylated regions of the soybean genome. We designed an *Agrobacterium* transformation preinoculation medium containing 5-azacytidine and 2-4-D to

partially address this question. In gene silencing studies in transgenic plants, one mechanism related to variation of transgene expression is correlated with the level of DNA methylation (reviews by Finnegan and McElory 1994; Matzke and Matzke 1995; Spiker and Thompson 1996). The cytosine-5-methyltransferases (DNA methyltransferases [Mtases]) inhibitor 5-azacytidine has been reported to increase expression of reporter GUS gene expression as well as re-activate gene expression in several different plants (Palmgren et al. 1993; Mandal et al. 1993). In addition, *Agrobacterium* mediated transformation of soybeans with the GUS reporter gene varies with different genotypes and environmental effects (Owens and Cress 1985; Savka et al. 1990). It was known that the soybean genome has a uniquely high content of heterochromatin (Hymowitz and Singh 1987) since it has 60% of repeate sequences scattered among single copy regions (Goldberg 1987). Soybean pachytene chromosomes indicate that over 35% of the genome consists of heterochromatin; the short arms of six of the 20 bivalents are completely heterochromatic (Singh and Hymowitz 1988). We observed that GUS expression could be significantly increased from 33% to 87% by the combined effect of 5-azacytidine and 2-4-D. PCR studies on GUS expression from transgenic soybean roots also suggests that the previously observed low level of GUS gene expression may be correlated with the level of DNA methylation. The main results of this study are presented in chapter 4.

Screening for *Phytophthora sojae* resistance in transgenic soybean roots

The conventional hypocotyl screening methods for *Phytophthora* is done by inserting a small piece of mycelium into the incision site in the hypocotyls of young soybean seedlings. This inoculation method will kill susceptible seedlings. A similar approach, but with inoculation of zoospores, is also effective (Eye et al. 1978). Smith et al. (1991) reported an in vitro assay for evaluation of

Phytophthora on soybean taproots, but this method is not useful for screening F2 populations because it is time consuming and expensive.

A highly efficient methods to produce larger numbers of transgenic soybean plants are not available. An *Agrobacterium rhizogenes* mediated transformation system was developed to verify putative cloned plant disease resistance genes. This method can also be used for F2 populations. Disease resistance in heterozygous or homozygous plants can be distinguished during segregation analysis in F2:3 individual lines since resistance is a dominant trait. Thus, an inoculation method for screening F2 populations is necessary to obtain information about phenotypic segregation (3:1). The main results of these studies are presented in chapter 5.

Dissertation organization

Chapters II, III, IV and V of this dissertation are presented in manuscript format. In Chapter II, I have described molecular cloning and characterization of yeast SNF2-like *Phytophthora* resistance genes from soybean. In Chapter III, methodology is presented for construction of genomic and c-DNA libraries from *Phytophthora* resistant soybean near isogenic line L85-3044. The construction of c-DNA library was assisted by a rotation graduate student, Mr. Chuanfa Jie . Chapter IV describes a high GUS expression efficiency for *Agrobacterium* mediated transformation procedure. This chapter also addresses that low efficiency of *Agrobacterium* mediated transformation may be due to unique features of the soybean genome. Chapter V describes an in vitro screening assay for *Phytophthora* resistance using transgenic soybean roots. The inoculation method was improved with the cooperation of Dr. X. B. Yang's group from the Plant Pathology Department of Iowa State University. Finally, chapter VI presents the general conclusions derived from these studies.

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CHAPTER II. CLONING OF A YEAST SNF2 HOMOLOGY FROM SOYBEAN

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ABSTRACT The SNF2 gene of yeast is a member of a large group of related proteins that exhibits a broad range of biological functions, such as gene-specific transcriptional activation, destabilization of reconstituted nucleosomes and transcriptional repression. We have cloned a homolog of the yeast SNF2 gene from soybean even though our intention was to clone disease resistance genes. Using a modified differential display, primers were designed from the P-loop region of plant disease resistance genes and were used in a differential display between two near isogenic lines that differed with respect to resistance to *Phytophthora sojae*, a damaging pathogen of soybeans. Polymorphic bands were identifed including a unique 0.7 kb band. This band was used as a probe to screen cDNA library from the resistant line (L85-3044). One of clones was identified as gmSNF2, a homolog of the SNF2 gene from yeast. The putative gmSNF2 protein has a leucine zipper motif, a ATP binding motif, and a leucine content of 11%. And it has a average 39.0% similarity to six putatine protein sequences of known plant disease resistance genes. Genetic complementation with a yeast SNF2 mutant indicates that the soybean gmSNF2 gene has SNF2

function. To test disease resistance function of gmSNF2 in soybean, an efficient transient assay was developed based on *Agrobacterium rhizogenes* mediated transformation. In this assay, *Phytophthora* susceptible plants develop a dark color upon infection with the fungus, while resistant plants do not. We found that introduction of the gmSNF2 gene into roots of cultivars Hark and Williams (susceptible lines) conferred resistance. We speculate that the action of gmSNF2 may be via free sugar molecules freed from fungal cell walls by soybean glucanases and thus initiates a sugar mediated signal transduction pathway for resistance to *Phytophthora sojae.*

The elicitor-receptor hypothesis states that pathogen avirulence genes specifically produce unique signal molecules, called elicitors, that are recognized only by the plant genotypes carrying the complementary disease resistance genes (1,2). In *Phytophthora sojae*, there are at least two classes of elicitor molecules. One class includes the oligoglucans (2, 3) and the other class is oligopeptides (4). The soybean β-glucan elicitor binding protein has been cloned in soybean (5), but it does not have any common conserved motifs of known plant disease resistance genes. The oligopeptides elicitor receptor protein also has been cloned and reported as a specific MAP kinase from parsley (6) but it does not have specificity to soybean.

In the early stages of the infection of soybeans by the fungal pathogen *Phytophthora,* soybean extracellular β -1, 3-endoglucanases attack the invading fungus releasing elicitor active carbohydrates from the *Phytophthora* cell wall (2, 7). The released β-glucan elicitors can bind to receptor proteins which have significant homology to glucanases (5). These elicitors lead to a host defense response, including phytoalexin and polyphenol production. The glucanases in

soybean not only release β-glucan elicitors with varying backbone chains but also release glucose and other sugars from fungal walls (8). The biological functions of these released sugars are not known. However, it has recently been reported that glucose and other sugars may play an important role in mediating signal transduction in plants (9). Plants fed various sugars or glyceollin produce immature trifoliolate leaves enhenced resistance to *Phytophthora sojae* race 1 in susceptible soybean cultivars Harosoy 63 and Harosoy (10). Added sucrose also enhances the expression of wound inducible potato inhibitor genes which decrease digestibility and nutritional quality of the leaf proteins (11).

We report cloning and characterization of a soybean gmSNF2 gene that has homology to the yeast SNF2 gene. This was done using a differential display utilizing a primer designed from the P-loop of plant disease resistance genes. The SNF2 gene of yeast is a member of a large group of related proteins (12) and SNF2 induces depression of the SUC2 (invertase) gene, and other glucose-repressible genes, in response to glucose deprivation (13). Members of the SNF2-like family in yeast exhibit a broad range of biological functions, such as gene-specific transcriptional activation, destabilization of reconstituted nucleosomes, and transcriptional repression (14). Based on our complementation test results, we hypothesize that the gmSNF2 may be involved with sugars freed from fungal cell walls by soybean glucanases, and somehow initiate a sugar-mediated signal transduction pathway for *Phytophthora* resistance. This gmSNF2 gene is likely the first cloned transcriptional activator that plays an important role in the sugar mediated signal transduction pathway related to the plant disease resistance.

MATERIALS AND METHODS

Plant and bacterial culture. The soybean *Phytophthora sojae* resistant near isogenic lines, Williams (susceptible) and L85-3044 (resistant), were provided by Dr. R. Nelson from USDA soybean germplasm collection center at the University of Illinois, Urbana, ILL. *Phytophthora sojae* race 5 and 25 were collected from Iowa soybean fields(33). The *Agrobacterium rhizogenes* strain K599 was provided by Dr. S.K. Farrand, University Illinois, Urbana, ILL. The disarmed binary plasmid p35S GUS INT which contains a GUS gene with an intron, and a NPTII selection maker gene between the right and left border regions was obtained from Dr. G. Vancanneyt, Institüt für Genbilogische Forschung, Berlin. This plasmid was used for transformation of soybeans in control experiments. GmSNF2 was subcloned into this plasmid by deleting GUS INT gene. The yeast SNF2 mutant used in the complementation test was kindly provide by Dr. Marian Carlson, Columbia University, NY. Soybean seeds were sterilized by chlorine gas and were then germinated under sterilie conditions. Methods for inoculation of soybean seedling with *Phytophthora* race 5 were described by Smith (15).

Nucleic acid procedures. RNA from soybean seedling roots was isolated as described previously (16). The first strand cDNA was synthesized by reverse transcriptases MLV according to Liang and Pardee (17) and purified using Wizard PCR kit from Promega Inc. The second strand was synthesized utilizing PCR, one primer of poly-T and the second degenerate primers from the P-Loop sequence of plant disease resistance gene or from the GLPLAL motif of RPS2 gene in *Arabidopsis*. The PCR reactions were set by using a PCR master mix that was consisted of 1 unit Taq polymerase, 5 pmoles of each primer, 1% (v/v) of dimethyl sulfoxide, 5 µl of 10X Mg-free thermophilic DNA polymerase buffer, 200

μM of each dNTP, 5 μl of 0.05 mM MgCl. The cycling parameters were as follows: 94°C for 5 minutes, 57° C for 2 minutes, 72° C for two minutes, then 35 cycles of 94° C for 30 seconds, 57°C for 30 seconds and 72°C for 2 minutes. The PCR products were separated in a 1% agarose gel and the polymorphic bands were purified and cloned into a pT7Blue vector of Novagen Inc. A cDNA lambda library was constructed from a soybean *Phytophthora sojae* resistant line L85-3044 (16). The clones from this cDNA library, present in the ZAP expression vector, were excised into a phagemid by the ExAssist helper phage from Stratagene Inc. and used for direct DNA sequence analysis from both directions.

Complementation test. *S. cerevisiae* SNF2 mutant strain (MCY 1250) was used as a host in the complementation test. A yeast/*E.coli* shuttle vector pYX112 was used for expression in the *S. cerevisiae* SNF2 mutant strain. This pYX112 vector has a TPI promoter, URA3 selection marker and a ARS/CEN plasmid. URA3 positive transformants were replica plated onto raffinose containing medium for the complementation test (20).

Transgenic roots. The *Agrobacterium rhizogenes* mediated transformation of soybean seedlings were based on modification of previous described methods (21). A single clone of *Agrobacterium rhizogenes* strain K599 with a disarmed binary plasmid p35S GUS INT, which contained a GUS gene with an intron and a NPTII selection maker gene between the right and left border regions, was used for transformation of control soybean seedlings. The gene gmSNF2 was subcloned into this binary vector after removing the GUS gene. This construct with the gmSNF2 insert was transformed into *Agrobacterium rhizogenes* strain K599. Transgenic roots were induced with this strain on soybean seedlings. After 3 weeks of culture, the newly-grown transgenic roots were removed and used for PCR analysis and screening for *Phytophthora sojae* resistance. The newly grown roots from control plants were also assayed for

β-glucuronidase (GUS) activity by incubation in 0.5 ml of 5-bromo 4-chloro 3 - indolyl β-D glucuronic acid (X-Gluc) for 12 hrs at 37°C (22). The protocol for screening of *Phytophthora* resistance in transgenic soybean lines was based on a coloration assay (23). To verify that hairy roots were indeed transgenic, PCR was done on isolates to determine the presence of the plasmid. Two conserved sequences in the GUS gene were also used as primers, and were: 5'-TTC TTT AAC TAT GCC GGG ATC CA-3' for the upstream sequence and 5'-CGA GTG AAG ATC CCC TTC TTG TT-3' for the downstream sequence.

RESULTS

To identify clones involved in pathogen resistance in soybean we performed a differential display of mRNA populations from two near isogenic lines of soybean, Williams (sensitive) and L85-3044 (resistant), differing with repect to resistance to *Phytophthora sojae*. The primers used on the two cDNA populations were designed from the degenerate P-loop sequences of known plant disease resistance genes and the poly-A tail (Fig. 1). The polymorphic bands were generated from 1st strand cDNA. The 0.7 kb polymorphic band could be reproduced using different Taq polymerase sources. The 0.7 kb polymorphic bands was subcloned into pT7Blue vector and the DNA sequence was determined from three different clones. The comparison of putative protein sequences of known plant disease resistance genes with a protein sequence from the differential display are presented in Fig. 2. This sequence possessed a putative P-loop domain and have sequence similarity of ca 50% on average when compared to three known plant disease genes in Fig. 2 (24). Southern analysis of genomic DNA with clone dds8 against genomic DNA from cultivars L85-3044 and Williams showed a minor polymorphism around 4.0 kb bands when digested

with restriction enzymes *Sal*I and *Hind* III. Thus, the identified polymorphic insert from clone dds8 was used as probe for screening libraries. The cDNA library was prepared from pooled cDNA from *Phytophthora* infected and uninfected roots of soybean line L85-3044 into the ZAP express EcoR1/CIAP vector. Seven positive cDNA clones were identified from this library. Here we only report the cDNA gene sequence that has homology to the yeast SNF2 gene (13,14).

The structure and sequence of cDNA gmSNF2 clone was obtained and showed an open reading frame (Fig 3). The cDNA sequence was 2451 bp with an open reading frame corresponding to a polypeptide of 768 amino acid residues and a predicted molecular mass of 8.7 kDa. 11.7% of total amino acids in the putative gmSNF2 protein consisted of leucine. A leucine zipper motifs (L-x(6)-L-x(6)-L-(6)-L) was identified between amino acids 586-607 and a highly conserved ATP binding motif (ILADEMGLGKTVQ) between amino acids 277-289 (14). In addition, the sequence possessed five putative N-glycosylation sites (red), nine N-myristoylation sites (yellow), nine protein kinase C phosphorylation sites (blue), twenty casein kinase II phosphorylation sites (bold) (Fig. 3). In addition, the sequence possessed similarity to DNA helicase regions (14). The hydrophobicity of this protein sequence is illustrated in Figure 4. No significant transmembrane domain were identified in the gmSNF2 sequence, thus it appears to be cytoplasmic. The Blastp+Beauty DNA sequence comparison of gmSNF2 with existing gene databanks produced a very broad range of proteins (probability range from 5.9e-92 to 2.5e-68) which were members of the gene family of transcriptional activators in yeast, such as Fun30p (P31380), SNF2 gene (M89907), and ISW1 (L27127). The comparison of gmSNF2 with yeast SNF2 gene is presented in Fig.5 and indicates a 50.1% similarity (39.95% identity) in the putative gmSNF2 protein between amino acids 254-791 of

gmSNF2. The gmSNF2 gene appears to satisfy the basic amino acids present in 5 conserved domains in the SNF2/SWI2 family (14). It was interesting to us that putative protein sequence of gmSNF2 had an average of 39.5% similarity (28.65% identity) to six known plant disease resistance genes, all which have leucine rich regions. Specifically, gmSNF2 had 37.31%(26.62%), 43.24%(29.73%), 37.59%(28.23%), 40.58%(28.78%), 39.57%(29.46%), 38.97%(29.11%) similarity (identity) to RPS2 in Fig. 6 (25,26), N(27), L6(28), Cf-2(29), Cf-9(30), and Xa-21 gene (31), respectively.

To determine if the gmSNF2 gene has SNF2 function in yeast, we transformed the gmSNF2 gene into a SNF2 mutant strain and determined if the SNF2 gene was complemented. The URA3$^-$ SNF2$^-$ mutant cannot grow on raffinose as a carbon source but can grow in yeast rich medium(13). We selected for URA3 prototrophy with a pXY yeast/*E.coli* shutter vector containing the gmSNF gene. The strain was also transformed with the vector pXY as a control. URA3$^+$ transformants carrying URA3$^+$gmSNF2 and control pXY were then replicated plated on raffinose medium. The yeast SNF2 mutant strain transformed with gmSNF2 gene grew on raffnose-containing medium, and the control did not (Fig. 7). These results suggest that the soybean gmSNF2 gene can provide SNF2 function in yeast.

We then determined if the gmSNF gene could complement gmSNF2 in a sensitive line of soybean using a transient expression system for *Phytophthora* resistance assay in soybean roots. Since an efficient method for producing transgenic soybean plants is not available, an *Agrobacterium rhizogenes* mediated transformation system was developed (21). Table 1 summarizes the pooled data from the *Phytophthora* inoculation test (with race 5 and 25) of transgenic soybean roots of L85-3044, William and Hark that were transformed with gmSNF2 plasmid construct and control vector p35S GUS IN. All susceptible

non-transgenic roots from Williams and Hark were given a score of 3 but all non-transgenic roots from resistant variety L85-3044 were scored as 1. Fig 8 shows that *Phytophthora* infected roots of Williams and L85. In table 1, several susceptible soybean roots transformed with gmSNF2 gene produced a colorless reaction, and when transformed with the control plasmid with only the GUS gene, still produced a susceptible reaction. This indicated that gmSNF2 can complement to Williams and Hark. However, some (ca 33%) susceptible roots can be scored as a 1 when transformed with just the vector p35S GUS INT. These results are explained as being due to a response of general defense-related plants proteins, such as chitinases and ribosome-inactivating protein trichosanthin, which accumulate after *Agrobacterium* infection (15). As positive control, *Phytophthora* resistant variety L85-3044 was also transformed with the same plasmid. Transgenic roots from resistant variety L85 produced a susceptible color reactions after a *Phytophthora* infection which can be attributed to co-suppression, a common phenomena in transgenic plants (32). The Southern blot analysis of various restriction enzyme digestion products form L85-3044 and its near isogenic line Williams showed there was only a small polymorphism. This 2.0 kb bands from BamH1 digestion was probed with EcoR1 fragment of gmSNF2 gene in Fig 9. A variance analysis of the assay data are shown in table 2, and indicates that the effects of plasmid on the color reactions are statistically highly significant. *Phytophthora* susceptible variety Williams and Hark transformed with gmSNF2 produced statistically significant resistance reactions. The effects of genetic background from the three varieties is also highly significant.

In this complementation assay, we have consistently observed that transgenic roots more than four weeks old, or when cultured on media containing

a high content of sucrose (range from 2% to 3%) will not respond to infection by *Phytophthora (*data not shown).

DISCUSSION

Phytophthora root and stem rot of soybean is one of the most destructive fungal disease in soybean-producing areas of the world, and it can result in a complete yield loss in some areas (33). It is important to clone the *Phytophthora* resistance genes in soybean as it may help in molecular marker assisted backcrossing programs, the design of new fungicides and our understanding the mechanism of the interaction between plant pathogens and the host. All reported plant disease resistance genes have been cloned by either transposon tagging or map based positional cloning (34, 35). Each approach has its advantages and disadvantages. Transposon tagging is dependent upon a transposable element system in the plant in question. But, some crop plants, such as soybean and wheat, have no cloned transposon tagging system. Map based cloning is dependent on the availability of closely linked flanking markers (36), which can be difficult to obtain, especially for polyploids species that have fewer polymorphic molecular markers.

The soybean (2n = 40) is regarded as a stable tetraploid with diploidized genomes (37). It has more than 60% of repeated sequence scattered among single copy regions (38) and its pachytene chromosomes indicate that over 35% of the genome consists of heterochromatin. The short arms of six of the 20 bivalents are completely heterochromatic (39). In addition, plant disease resistance genes frequently occur in tightly linked clusters or as true alleles (40). Thus, it is likely that map based cloning of genes from soybeans would be more difficult than in other simple diploid crop species, even if closely linked markers were available

(36, 41, 42). Since more than two copies of a gene may be present in the genome, recombination between the polymorphic linked markers and a target gene may be inhibited by its location around heterochromatin(43, 44), thus linkage information may be biased. Thus, it may be a considerable amount of labor to walk to the target gene in the soybean genome. In recent years, several plant disease resistance genes have been cloned by either transposon tagging or map based positional cloning. Their molecular structures and functions of these plant disease resistance genes have been the subject of several reviews (24,34,35,45, 46,). Plant disease resistance genes possess similar molecular motifs such as leucine rich repeats (LRR) involved in protein-protein interactions or binding of peptide ligands (47), nucleotide binding sites (NBS) and P-loop amino acid sequence which bind specifically to phosphate nucleotides in energy reactions (48). It has been proposed that plant disease resistance may have a common molecular mechanism and involve a signal transduction pathway (24, 34, 46, 49). It is not yet known what role the consensus motifs play in the signal transduction pathway of plant disease resistance. But, due to the conserved sequences in these genes, several groups have reported finding disease resistance gene homologs in soybean (50, 51).

Arbitrary primed PCR fingerprinting and differential display resolved on sequence gels or agarose gels have been used to detect differentially expressed RNAs (17, 20). To increase the efficiency for expression analysis of multigene families, Fisher, et al (52) proposed a restriction fragment length polymorphism-coupled domain directed differential display. Consequently, we have used PCR primers designed from the conserved motifs of known disease resistance genes, specifically the P-loop sequence in a differential display. By identifying polymorphic clones from the differential display and screening a cDNA library, we cloned a gmSNF2 gene and several other plant disease resistance related

genes, including a peroxidase gene and a cadmium-transporting ATPase. Our differential display results indicate that designing primers from known conserved regions provides an alternative methods for the cloning of genes from polyploid species. However, the disadvantage of above methods may be that some plant disease resistance genes do not have common conserved motifs, such as the Mlo gene from barley, which confers a broad spectrum resistance to the fungal pathogen, *Erysiphe graminis* f.sp.*hordei* (53). Mlo has six membrane-spanning helices and two of casein kinase II sites. Gene NPR1 from *Arabidopsis thaliana* controls systemic acquired resistance that contains unique ankyrin repeats (54). The ankyrin repeats consensus motif was found in various groups of proteins involved in cell structure, transcriptional regulation, cell differentiation, and enzymatic and toxic activities (55). Screening primers from polymorphic RFLP probes in a differential display should overcome the above shortcomings. We have also cloned another SNF2 gene family member from soybean by this approach, as well as a GRR1 yeast homolog.

The structural analysis of gmSNF2 shows that it not only has similar motifs of SNF2/SWI2 but also has a 40% similarity and 28.6% identity around leucine rich region to 6 known plant disease resistance genes. These six known plant disease resistance genes also have GTP/ATP binding sites or P-loop amino acid sequences and kinase motifs. The gmSNF2 gene also has a putative ATP binding site and kinase motifs. The yeast complementation test shows that it has SNF2 function in a yeast snf2 mutant. Thus, this finding would suggest that gmSNF2 has transcriptional activator function. The complementation of gmSNF2 in soybean *Phytophthora* susceptible varieties produces resistance reactions. This finding would suggest that gmSNF2 may play a role in the soybean *Phytophthora* resistance signal transduction pathway. In the early stages of the infection of soybeans by the fungal pathogen *Phytophthora*, soybean

extracellular β -1, 3-endoglucanases attack the invading fungus and release elicitor active carbohydrates from the *Phytophthora* cell wall (2, 7). This soybean endoglucanases not only releases elicitors but also glucose and other sugars from the fungal cell wall (8). These released elicitors lead to a host defense response. including phytoalexin and polyphenol production. The biological function of the glucose as well as other sugars released from fungal wall was not known. It has been recently reported that glucose as well as other sugars may play an important role in mediating signal transduction in plants (reviewed by Smeekens and Rook, 9). It is also known that after a certain seedling age (when the products of photosynthesis reach a certain threshold level), soybean seedlings are no longer susceptible to infection by *Phytophthora.* It was reported that immature trifoliolate leaves from plants fed various sugars or glyceollin promote resistance to *Phytophthora sojae* race 1 in both soybean Harosoy 63 and susceptible cultivar Harosoy (50). We have also consistently observed that transgenic roots of more than four weeks old, or when cultured on media containing a high content of sucrose (range from 2% to 3%) do not respond to infection by *Phytophthora.* Sucrose was reported to enhance the expression of wound inducible potato inhibitor genes that were the inducible defensive chemicals of plants which protect against herbivores by decreasing the digestibility and nutritional quality of the leaf protein (11).

Budding yeast preferentially utilize fermentation of glucose and genes involved in carbon metabolism pathways, including SUC2, which are repressed when sufficient levels of glucose are present in the environment (56). Interestingly, it was reported that the accumulation of pathogenesis-related protein transcripts (PR-Q) and repression of photosynthetic gene transcripts (chlorophyll α/β binding protein) were also inversely correlated and required the same threshold level of hexoses for induction and repression in tobacco plants

(57). The study of transgenic tobacco plants with yeast-derived invertase showed that spontaneous necrotic lesions similar to hypersensitive response were caused by avirulent pathogen infections (57). Thus, we hypothesize that the gmSNF2 may sense the released free sugar molecules from fungal cellwalls and initiate a sugar mediated signal transduction pathway for *Phytophthora* non-race specific resistance in soybean.

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Table 1. Color assay for scoring transgenic roots that were inoculated with *Phytophthora sojae*

Variety Name	Vector Type	Color Scoring		
		1	2	3
W	NA	0	0	22
W	ck	3	6	6
W	gmSNF2	3	0	0
L	NA	15	0	0
L	ck	9	9	3
L	gmSNF2	3	6	6
H	NA	0	0	21
H	ck	3	3	12
H	gmSNF2	9	6	15

Variety name: W= William, L=L85-3044, H=Hark

Vector type: ck = p35S GUS INT. NA = no vector used

Color Scoring: 1= resistant, 3 = susceptible,

2 = intermediate type.

Table 2. Variance analysis of color assay from transgenic soybean roots that were inoculated with *Phytophthora sojae*

Source	df	SS	F-Value
Var(V)	2	23.76	29.25 **
Vct	2	4.11	5.06 **
V * Vct	4	28.77	17.71 **
Error	151	61.34	
Total	159	119.49	

V= varieties for L85-3044, William and Hark; Vct = plasmid vector for gmSNF2 and control plasmid vector p35S GUS INT and plants transformed with no vector; ** = highly significant at $P \leq 0.01$; df = degree of freedom; SS = type III sum of squares.

FIGURE LEGENDS

Figure 1. Polymorphic bands from the PCR reaction using primer designed from *Arabidopsis* RPS2disease resistance gene P-loop differential display

Figure 2. The comparison of protein sequences of known plant disease resistance genes with one of putative protein sequences of clones from differential display .

Figure 3. The putative protein sequence of the gmSNF2

Figure 4. The hydrophobicity of protein sequence of the gmSNF2 and SNF2

Figure 5. The comparison of putative protein sequence of gmSNF2 with yeast SNF2 gene

Figure 6. The comparison of putative protein sequence of gmSNF2 with rps2 gene from *Arabidopsis thaliana* that confers resistance to the bacterial pathogen *P. syringae*

Figure 7. Yeast complementary test of gmSNF2 in snf2 mutant in yeast. The growth of transformed clones showed that only the strain with gmSNF2 gene from soybean grew on raffinose medium.

Figure 8. The *in vitro* screen soybean roots. This picture shows that *Phytophthora* infected roots of L85-3044 produced a colorless reaction but susceptible line Williams produced a dark color reaction

Figure 9. The southern blot analysis of various restriction enzyme digestion products form L85-3044 and its near isogenic line William showed there was only a small polymorphism between L85-3044 and William around ca 2.0 kb bands of restriction enzyme BamH1 digestion when probed with EcoR1 fragment of gmSNF2 gene

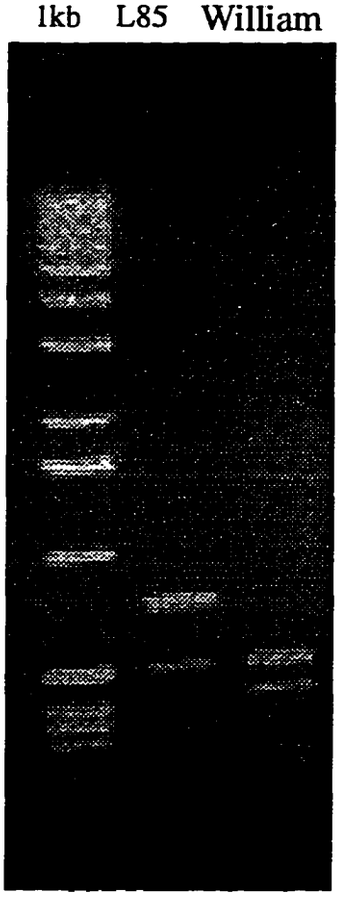


Figure 1.

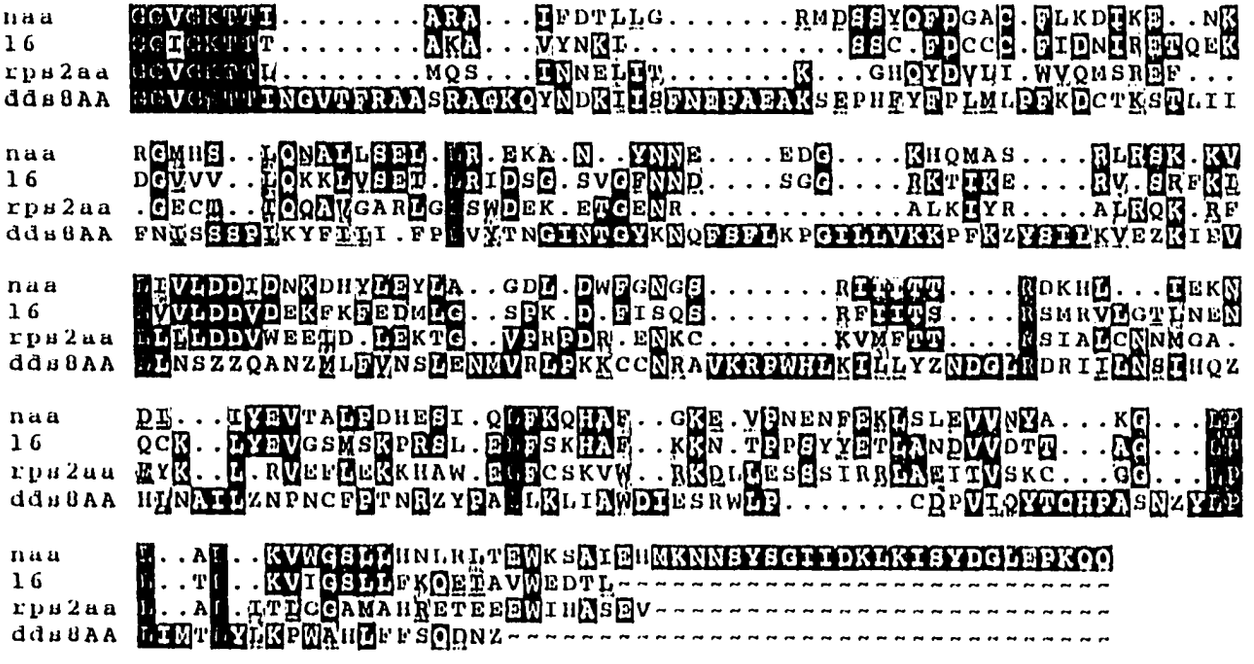


Figure 2

THLIALSTMKPELYEISDDEWENHSFKPSRVLKRPRTSSPPSPPPVESFAYTSTSKV
DVSSENDDDSDCVEIAPEAANFRQNLDDLEDADVDDEPVPASRGRRFIIDEEEEEE
DGEEENGGRDGHLAELYDVESSEEEVVEEEVEELNENDVVGRALHKCARISAEL
KGELFGSSGTACERYSEVESSSVRIVTQEDVDVARGSEEDSGFKPLLKPYQLVGV
NFLLLLYRKGIGGAILADEMGLGKTVQAITYLTLLKHLHNDSGPHLIVCPASVLEN
WERELKRWCPSFSVLQYHGAGRAAYCKELNSLSKAGLPPPFNVLLVCYSLFERH
SAQQKDDRKILKRWRWSCVLMDEAHALKDKNSFRWKNLMSVARNANQRLMLT
GTPLQNDLHELWSLLEFMLPDIFATEDVDLKKLLNAEDGDLIGRMKSILGPFILRR
LKSDVMQQLVPKIQQVEYVIMEKQQETAYKEAIEEYRAVSQARMEKCSNLNSKS
VLEVLPRRQINNYFVQFRKIANHPLLIRRIYNDEDVIRFARKLHPIGAFGFECTLDR
VIEELKNYNDFCIHRLLLHYGVNDRKGILPDKHVMLSAKCRALAELLPSLKEGG
HRALIFSQWTSMLDILEWTLDVIGLTYKRLDGSTQVAERQTIVDTFNNDTSIFACL
LSTRAGGQGLNLTGADTVVIHDMDFNPQIDRQAEDRCHRIGQTKPVTIYRLVTKG
TVDENVSEIAKRKLVLDAAVLESMEEINEGDMPEKTMGEILSAILLNNIDLRKTV

Figure 3

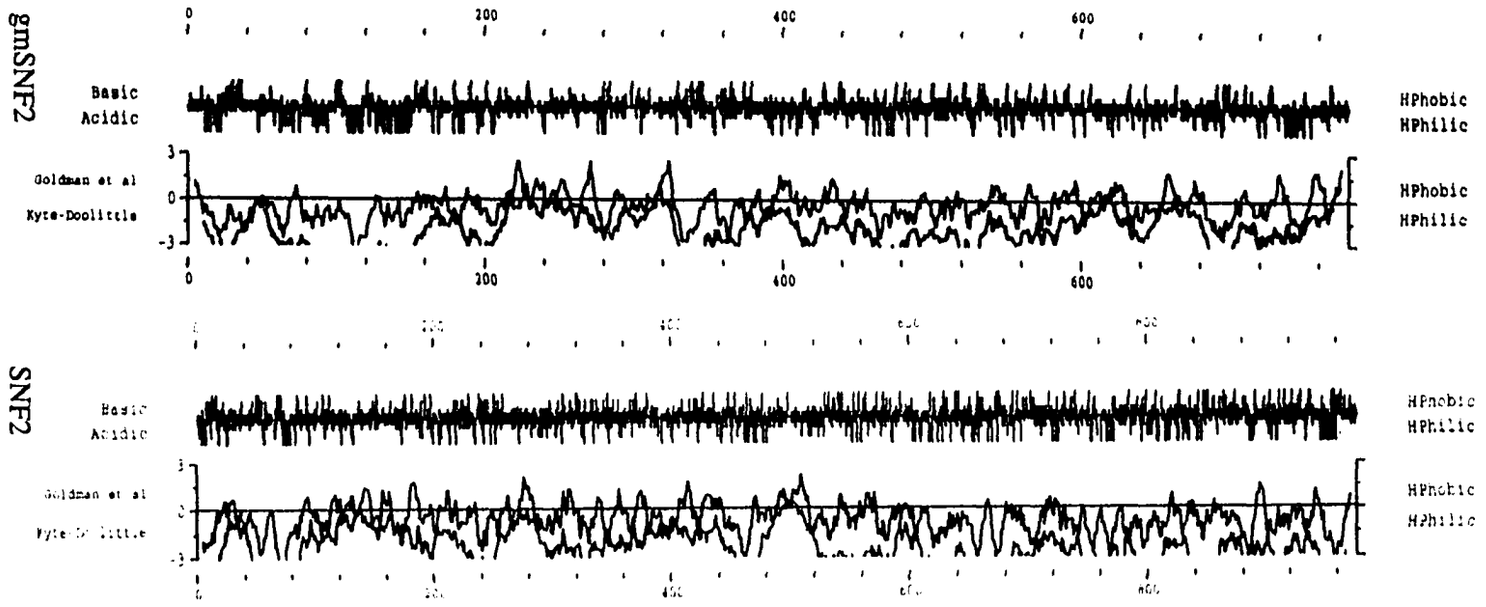

gmSNF2
Basic
Acidic
HPhobic
HPhilic
Goldman et al
Kyte-Doolittle
3
0
-3
0
200
400
600
HPhobic
HPhilic
SNF2
Basic
Acidic
HPhobic
HPhilic

Figure 4

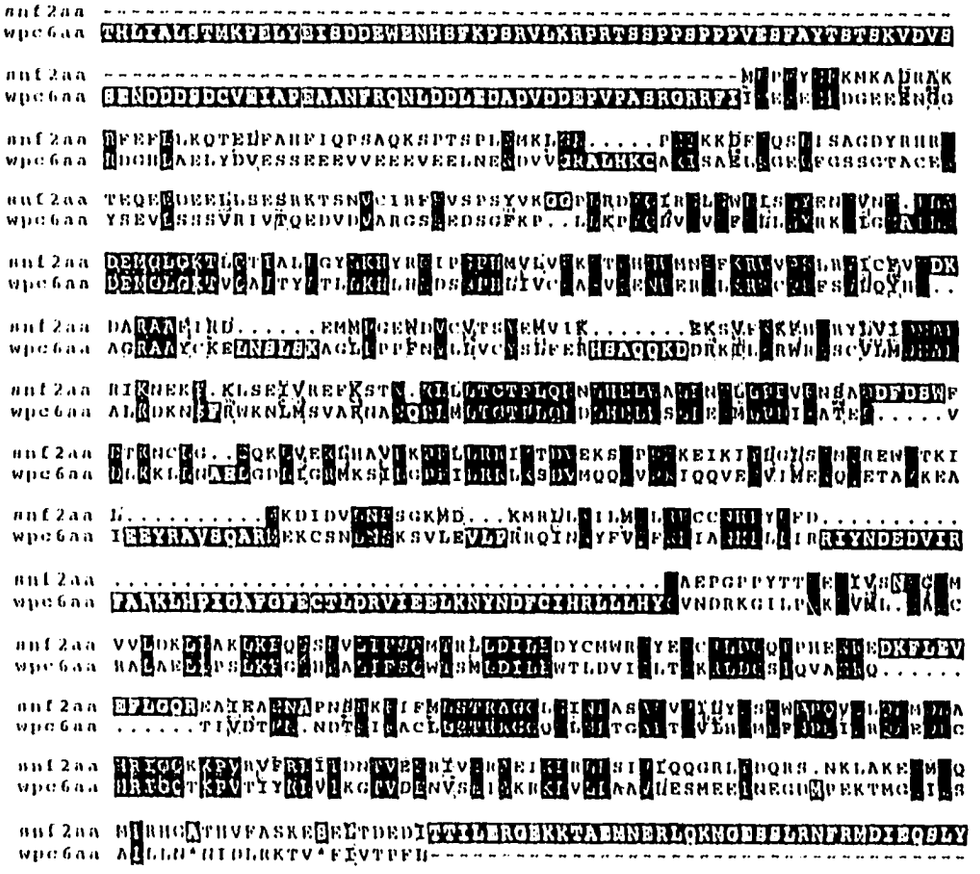


Figure 5

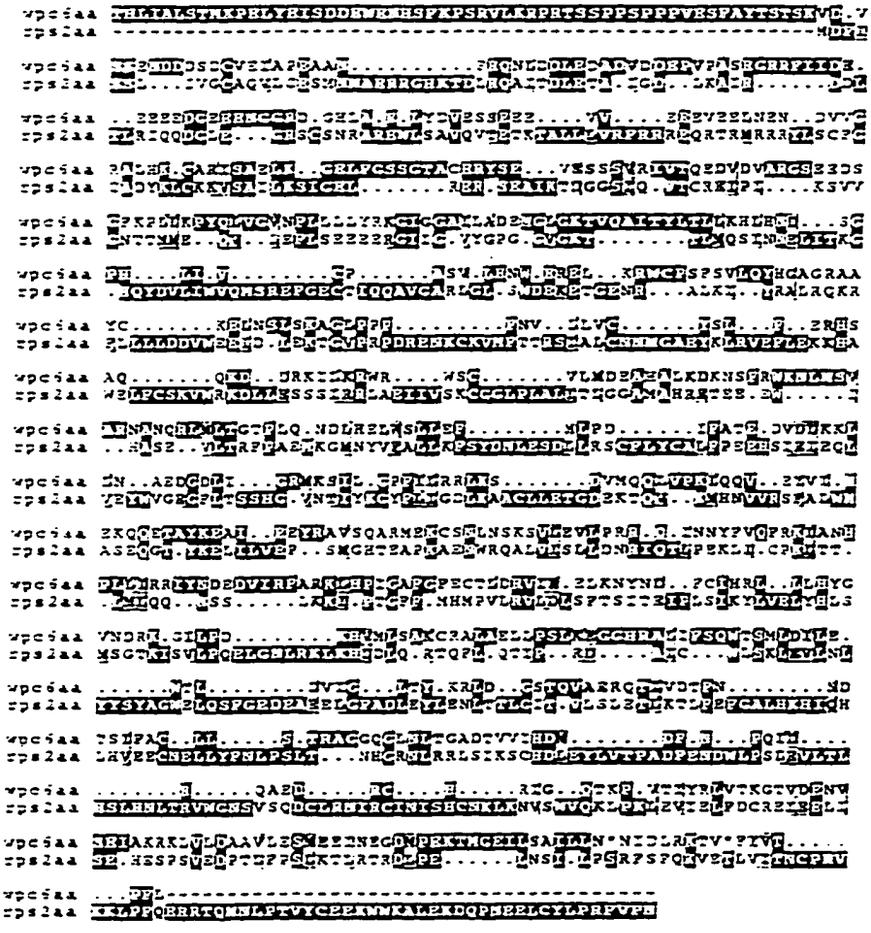
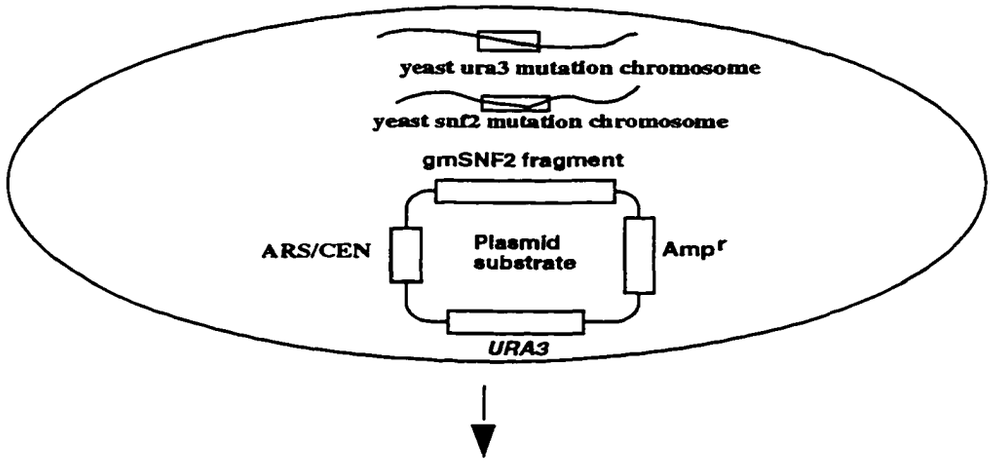


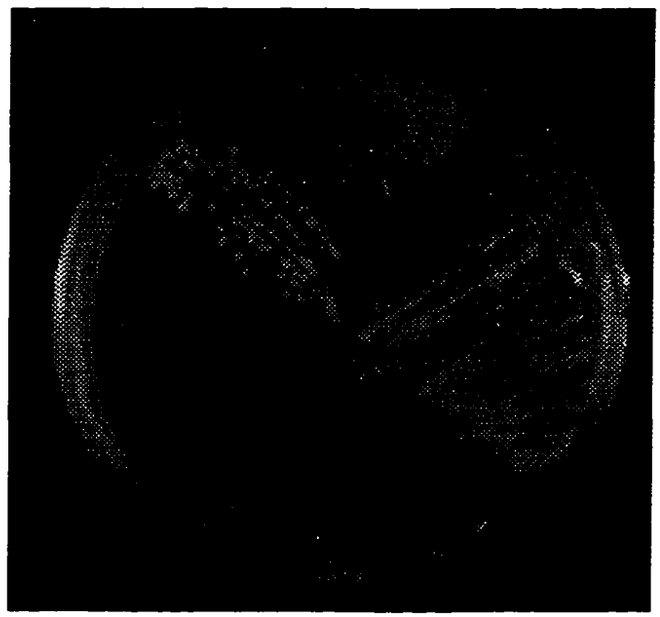
Figure 6



Complementation test transformed yeast snf2 mutant cells on -URA3 medium with raffinose source

gmGRR1

snf2 mutant cells



gmSNF2

vector control

Figure 7

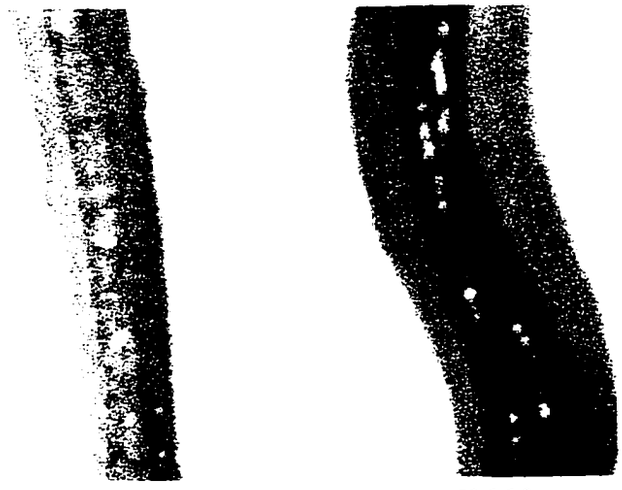


Figure 8

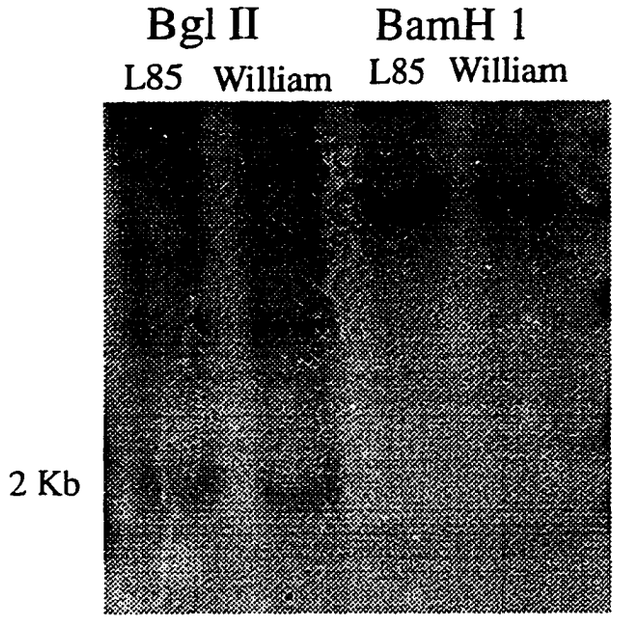


Figure 9

CHAPTER III. CONSTRUCTION OF A SOYBEAN GENOMIC & ROOT cDNA LIBRARY FROM A *PHYTOPHTHORA* RESISTANT LINE L85-3044

A manuscript to be submitted to Soybean Genetics Newsletter

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Introduction: Genomic (lambda, BAC and YAC) and cDNA libraries have been prepared from soybeans, but it is frequently necessary to have a library that is from a specific cultivar. In this respect, we have prepared both a genomic and cDNA library from a soybean line L85-3044 that possesses the *Phytophthora* resistance genes, and this line is a near isogenic line to Williams (Diers et al., 1992). An advantage of these libraries is that they were prepared from size fractionated DNA populations. Consequently, the insert size is consistantly large. Finally, the clones from the cDNA library were prepared in a pBK-CMV phagemid *in vivo* , thus putative genes can be expressed in both prokaryotic and eukaryotic cells.

Materials and Methods: Plant materials used were soybean line L85-3044, obtained from Dr. R. Bernard from University of Illinois soybean stock center. *E. coli* XL1-Blue MRA was used as the host strain for the genomic library and XL1-Blue MRF was used for construction of the cDNA library. *E. coli* strain XLOR and EXAssist helper phage were used for *in vivo* excision of selected lambda clones from the cDNA library and placed into a pBK-CMV phagemid

from the ZAP expression vector. For construction of the soybean genomic library from L85-3044, about 10 grams of fresh leaves were harvested from the greenhouse and genomic DNA was carefully isolated according to Doyle's CTAB methods (Doyle, et al., 1990). The quality and the size of DNA was estimated by pulse field gel electrophoresis (CHEF-DRII system, BIO-RAD) in 0.8% GTG agarose (FMC BioProducts, Rockland, ME) and 0.5 x TBE buffer at 10°C for 22 hours at 200 volts. The initial time was 1 second and the switch time was 50 seconds. DNA fragment sizes greater than 200 kb were used for restriction enzyme partial digestion and purified from the gel according to Chen, et al. (1994). About 50 μg of the DNA was digested and extracted with phenol/chloroform (3:1), precipitated with ethanol, and the digested genomic DNA products were incubated with Klenow reagent to fill-in the first two nucleotides of the *Sau*3A-compatible sites (Zabarovsky, et al., 1986). Lambda replacement vector, FIX II/*Xho*1 (Stratagene Inc., La Jolla, CA 92037) was used for construction of the genomic library and packaged with MaxPlax Lambda Packaging Extract (Epicentre Technologies Inc. Madison, WI 53713) according to manufacture's instruction. The XL1-Blue MAR (P2) was used as the host bacterial strain for the genomic lambda library.

For construction of soybean cDNA root library from L85-3044, soybean seeds were sterilized with chlorine gas and germinated on paper for about a week. The roots were inoculated with *Phytophthora* race 5 (Smith, et al., 1991). The pathogen isolate *Phytophthora soja* race 5 was provided from Dr. X.B. Yang of Iowa State University. Both inoculated and non-inoculated soybean roots were used for isolation of total RNA by TRIzol reagent (GIBCOBRL Life technologies, Inc. Gaithersburg, MD 20884-9980) according to the manufacture's instruction. The DNase treated total RNA was used for isolation of total mRNA by the PolyATract mRNA isolation system IV (Promega Inc., Madison, WI 53711-5399)

according to the manufacture's instructions. The mRNAs isolated from inoculated and non-inoculated soybean roots were pooled for synthesis of cDNA. The cDNAs were synthesized by Super Script Choice system (GibcoBRL Life Technologies Inc.) according to instructions. After synthesis with ^{32}P-dCTP to label the second strand cDNA, the size of cDNA was estimated by gel electrophoresis and by radiography using Southern blot analysis (Sambrook et al., 1989). The double stranded blunt end cDNAs were ligated to an *Eco*R1 adapter and then phosphorylated by T4 polynucleotide kinase. The phosphorylated ^{32}P-dCTP labeled *Eco*R1-adapted cDNA fragments were size optimized with column chromatography using a cDNA size fraction column (GibcoBRL Life Technologies Inc.). The amount of cDNA from the size fractionation column was determined by the amount of acid-preceptible radioactivity (Cerenkov counts, CMP) of the second strain cDNA from the washed filter. The ligation products and predigested ZAP Express *Eco*R1/CIAP vector DNA were finally packaged into lambda by Gigapack III Gold Packaging Extract (Stratagene Inc.) according to the instruction manual. The host strain XL1-Blue MRF' was used for the cDNA library.

Results and Discussion: To construct a genomic lambda library, high molecular weight DNA must first be isolated free from polysaccharides and other contaminants. Usually, the maximum size of DNA isolated from fresh leaves is about 50 kb or less (Chung et al., 1994) but are poorly suited for preparation of a genomic library (Slightom et al., 1993). We have previously reported a method for isolation of high molecular weight DNA from soybean leaves (Chen, et al., 1994), and this method was used to construct a soybean genomic cosmid library from the *Phytophthora* resistant line L85-3044.

Figure 1 shows the leaf genomic DNA separated by gel electrophoresis. Also shown are the ligation products of soybean L85-3044 genomic DNA *Sau*3A partial digestion to the lambda vector. Lanes 1 to 5 are from CHEF gel electrophoresis of soybean DNA in 0.8% GTG agarose. Lane 1 is the lambda concatemers used as DNA molecular weight size standards. Only DNA with fragments greater than 200 kb (lane 5) were used for construction of the library. Lane 6 shows the FIX II/*Xho*1 vector without ligation and lanes 7 and 8 are the ligation products of partially digested DNA and vector. The average number of recombinant phages of this lambda genomic library is 8.5×10^5. According to Slightom's calculation the theoretical recombinant phage numbers for a soybean genomic library should not less than 2.4×10^5 if 0.99% probability is desired. The average insert size is about 17 kb, and the soybean genomic size is 8.7×10^8 bp (Slightom et al., 1993). We isolated 20 positive lambda clones from this library by screening with a single probe from 7 plates (about 45,000 plaques per plate) in one of our experiments.

Another genomic library of L85-3044 was also constructed with lambda GEM-12 *Xho*1 half-site arm vector (Promega, Inc.). However, the packaged phage titration of this library was significantly lower than the lambda FIX II/*Xho*1 library. Both bacterial host strains were *mcr*A$^-$ and *mcr*B$^-$, allowing propagation of genomic DNA clones containing methylated cytosine residues. But the bacterial strain used for Lambda GEM-12 *Xho*1 half-site vector was *rec*D$^-$, which reduces recombinational loss but does allow certain eukaryotic clones to grow. Thus, this may result in a lower clone titration compared with the library constructed with Lambda FIX II/*Xho*1 vector. The constructed soybean genomic library from line L85-0344 from lambda FIX II/*Xho*1 vector is sensitive to P2 inhibition selection, that is, only recombinant phage grow on bacterial strains containing the P2 phage. This is due to the fact that wild type lambda phage

contain active *red* and *gam* genes on the stuffer fragment and are unable to grow on host bacterial strains that contain P2 phage lysogens. This characteristic significantly reduces the background of non-recombinant phages in the library. In addition, the lambda FIX II polylinker used in this library allows the isolation of flanking T3 and T3 promoters as an intact cassette after digestion with *Not*1. The T3 and T7 promoters flanking the insertion sites can also be used to generate end-specific probes for used in chromosome walking and restriction mapping. Figure 3 shows the flanking restriction enzymes sites around the *Xho* I site that soybean genomic DNA fragments were ligated into the Xho 1 partial fill in site in lambda FIX vector.

For a Soybean L85-3044 root cDNA library, it is important to have large sized fragments. The size of cDNA after second strand synthesis was determined by gel electrophoresis and by radiography through Southern blot analysis. The data in Figure 2 show the Southern blot analysis of the pooled random samples of ^{32}P-dCTP labeled second strand cDNAs before size fraction column chromatography. The cDNA fragments range from 0.5 to 7 kb with the major fraction ranging from 1 to 4 kb. To avoid incorporating small sized cDNA fragments and EcoR1 adapters into the cDNA library, we used size fractionation column chromatography to purify cDNA ligation products with EcoR1 adapter. Table 1 shows the amount of cDNA from the size fractionation column by calculation from the amount of acid-perceptible radioactivity (Cerenkov counts, CMP) of the second strand. We pooled fractions 2 through 8 and ligated the phosphorylated EcoR1-adapted cDNA fractions into our lambda vector (predigested ZAP Express EcoR1/CIAP DNA) for packing this cDNA library. We observed an average phage number of 3.6×10^5. The theoretical phage number should not be less than 1.8×10^5 if we assume a 0.99% probability. We isolated 20 positive cDNA clones from this cDNA library by screening with a single probe

from eight plates (about 45,000 plaques per plate) in one of our experiments. And, the fragment size of the cDNA inserts ranged from 0.8 to 3.4 kb in this experiment.

Purified clones resulting from a screen of this cDNA library, as well as random pooled clones from this library, can be excised into a pBK-CMV phagemid *in vivo* using the interference resistant helper phage XLOLR (Stratagene Inc.). Figure 4 shows where the soybean cDNA library was cloned into the *Eco*R1 site in the multiple cloning site (MCS). The insertions in pBK-CMV can be expressed in both prokaryotic and eukaryotic cells because the vector contains both the *lac*Z promoter for prokaryotic expression and CMV promoter for eukaryotic expression. The gene product (fusion protein of β-galactosidase) of the selected clone or random pooled clones of this cDNA library can be expressed in *E. coli* by adding 0.2 to 1 mM of IPTG in the liquid LB culture. We checked the expression of the random pooled cDNA clone samples from this cDNA library in *E coli* and observed 16 blue clones out of 210 white recombinant expression clones (92.4% expression of recombinants). To increase the expression level in eukaryotic cells, the *lac*Z promoter can be removed by *Nhe*I and *Spe*I digestion of the excised phagemid, followed by re-legating of the intact cassette and then selecting clones which have lost the 200 bp promoter fragment. The eukaryotic expression function of clone(s) of this library allows either transient or stable transfer of selected clones (target genes) directly into mutant or complementary cells or tissue for verification of the gene candidates.

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Table 1. Analysis of amount of cDNA from a size fractionation column.

Fraction No.	Volume(μl)	Accumulated Volume(μl)	Cerenkov Counts (CPM)	Amount of cDNA(ng/μl)
1	127	127	36	0.029
2	99	226	42	0.03
3	37	263	33	0.0269
4	38	301	29	0.0237
5	29	330	41	0.0335
6	37	367	722	0.59
7	36	403	5369	4.39
8	14.5	417.5	6324	5.169
9	35	452.5	19988	16.34
10	27	479.5	27969	22.86
11	37	516.5	36455	28.8
12	37	553.5	39261*	32.09

*The Cerenkov counts data after the accumulated volume of 550 μl cut off point is not recorded in this table.The amount of ds cDNA (ng) from the cDNA size fraction column was caculated based on the following: The amount of ds cDNA (ng)= [(Cerenkov cpm) x 2 x (4 pmol d NTP/pmol dCTP) x (1000 ng/μg ds cDNA)]/ [SA(cpm/pmol dCTP) x (1515 pmol dNTP/ μg ds cDNA)]. SA is the specific activity of the radioisotope in the first or second strand cDNA products. The regression equation for amount of cDNA (Y) with the log value of Cerenkov counts (X) in this experiment is: Y=2.28+0.104 X (r=0.79) in this experiment.

FIGURE LEGENDS

Figure 1. CHEF gel electrophoresis of soybean total genomic DNA isolated from leaves as well as the ligation products of partially digested genomic DNA. Lanes 1 to 5 are from soybean total genomic DNA in 0.8% GTG agarose. Lane 1 is the lambda concatemers used as DNA molecular weight size standards. Lanes 6 to 8 are 1% agarose gel separated ligation products of soybean L85-3044 genomic DNA *Sau*3A partial digestion products to the lambda vector. Line 6 shows the FIX II/*Xho*1 vector without ligation and Lane 7 and 8 are the ligation products of partially digested DNA and the vector.

Figure 2. Southern blot analysis of the pooled random samples of ^{32}P-dCTP labeled cDNAs before size fractionation with column chromatography. The cDNA fragments range from 0.5 to 7 kb with the major fraction ranging from 1 to 4 kb.

Figure 3 The soybean genomic DNA was partially digested with *Sau* 3A and replaced into *Xho I* partial fill in site. The restriction enzymes flanking around the *Xho I* are the enzymes that can cut soybean genomic insert fragment out from the Lambda FIX vector.

Figure 4. cDNA library was cloned into the *EcoR1* site in the multiple cloning site (MCS). The positions of restriction enzyme cutting sites, promoters and terminators of lac Z and CMV , replication origins of f1, CoE1 and SV40, lacZ gene, SV40 poly A terminate signal, and selection gene G418 for neomycin/kanamycine resistance were also illustrated in the map. This figure was adapted based on the pMK-CMV phagemid map of Stratagene Inc.

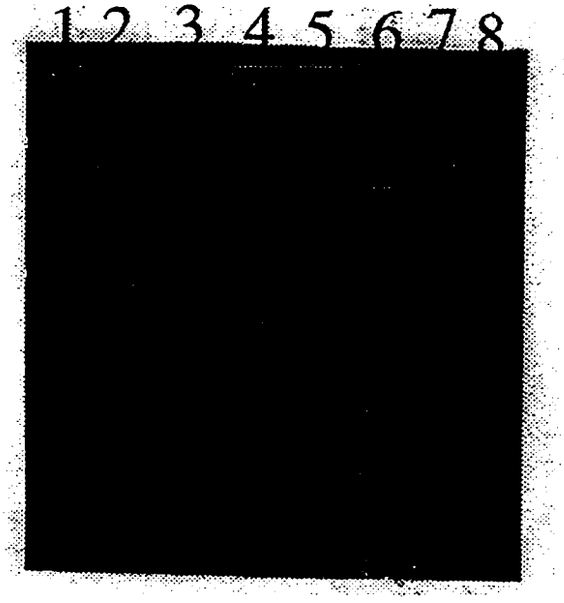

Figure 1

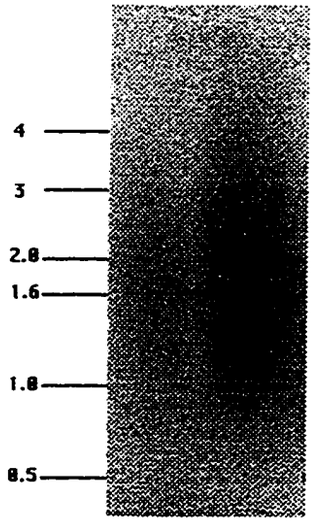

Figure 2

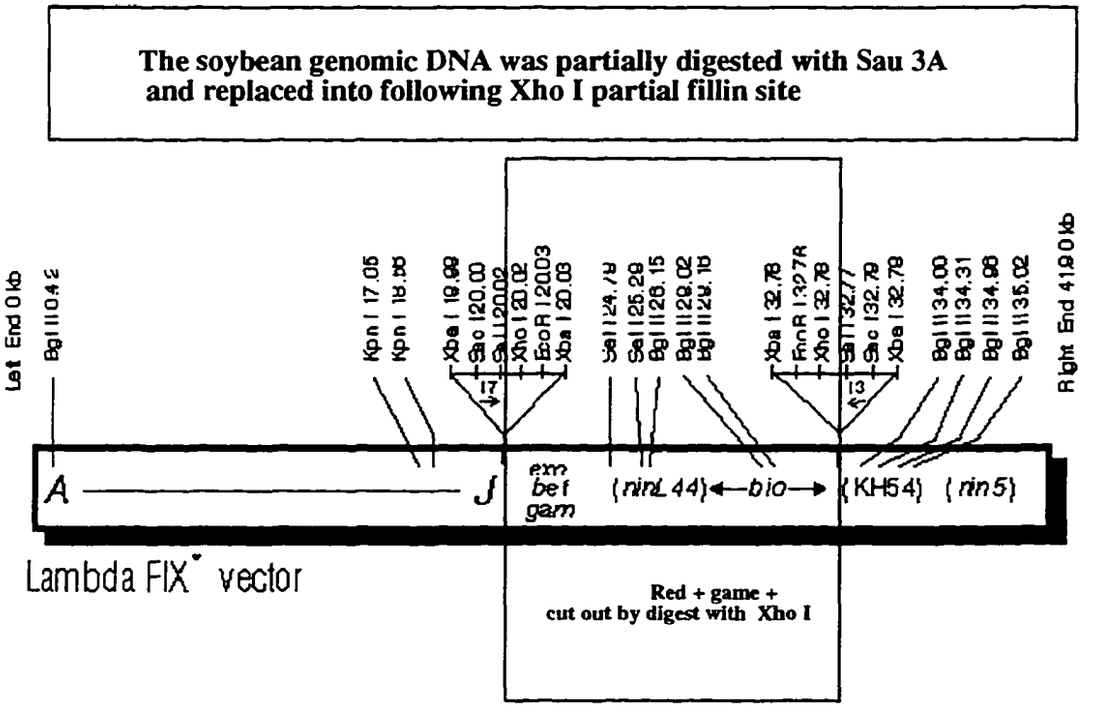
The soybean genomic DNA was partially digested with Sau 3A and replaced into following Xho I partial fillin site
Left End 0 Kb
Bgl II 0.42
Kpn I 17.05
Kpn I 18.00
Xba I 19.98
Sac I 20.00
Sal I 20.02
Xho I 20.02
EcoR I 20.03
Xba I 20.03
Sal I 24.79
Sal I 25.29
Bgl II 26.15
Bgl II 28.02
Bgl II 28.10
Xba I 32.78
EcoR I 32.78
Xho I 32.78
Sal I 32.77
Sac I 32.79
Xba I 32.79
Bgl II 34.00
Bgl II 34.31
Bgl II 34.98
Bgl II 35.02
Right End 41.90 Kb
17
13
A
J
em
bef
gam
{ninL44}
bio
{KH54}
{nin5}
Lambda FIX® vector
Red + game +
cut out by digest with Xho I

Figure 3

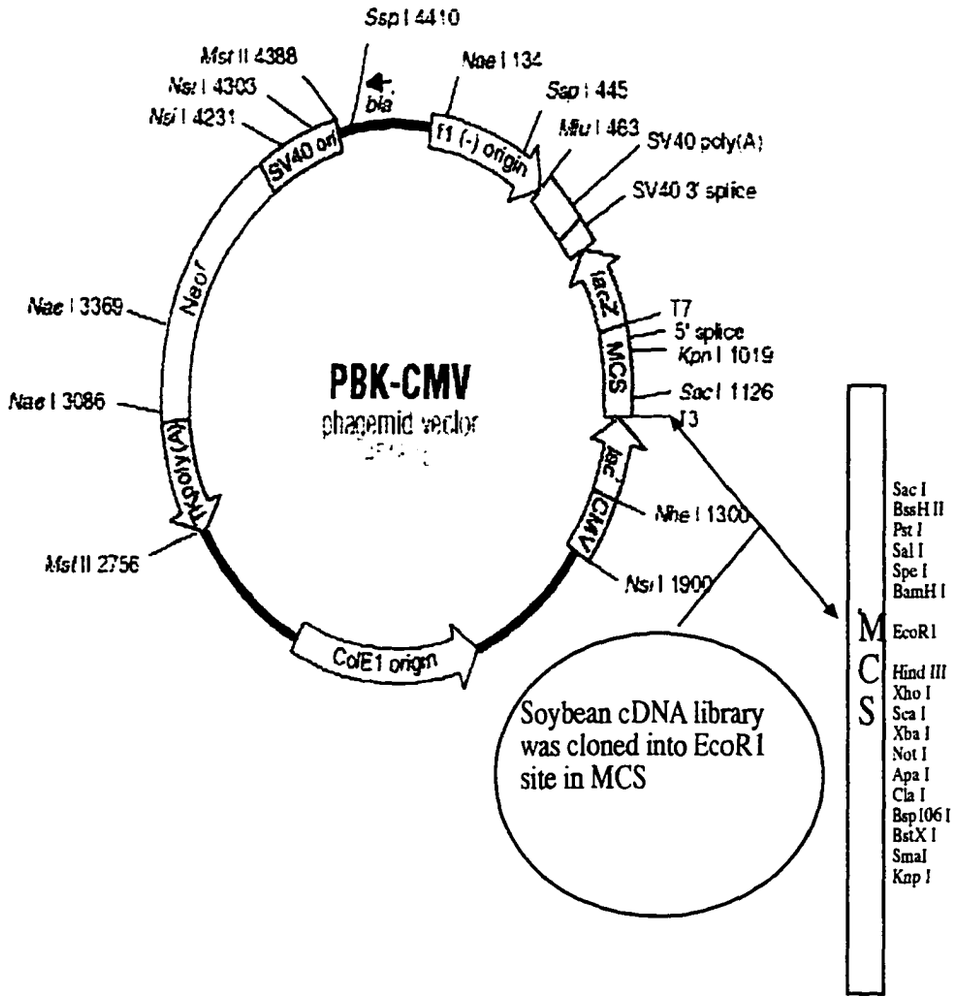
Ssp I 4410
Mst II 4388
Nae I 134
Nsi I 4303
Sap I 445
Nsi I 4231
bla
f1 (-) origin
Mlu I 463
SV40 poly(A)
SV40 ori
SV40 3' splice
lacZ
T7
5' splice
MCS
Kpn I 1019
Nae I 3369
Nae'
Sac I 1126
PBK-CMV
phagemid vector
Nae I 3086
KAN/NEO
lac
CMV
Nhe I 1300
Mst II 2756
Nsi I 1900
ColE1 origin
Soybean cDNA library was cloned into EcoR1 site in MCS
M
C
S
Sac I
BssH II
Pst I
Sal I
Spe I
BamH I
EcoR1
Hind III
Xho I
Sca I
Xba I
Not I
Apa I
Cla I
Bsp106 I
BstX I
SmaI
Kpn I

Figure 4

CHAPTER IV. INCREASED EFFICIENCY OF *AGROBACTERIUM RHIZOGENES* MEDIATED TRANSFORMATION OF SOYBEAN SEEDLINGS

A manuscript to be submitted to Plant Science

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Abstract

Agrobacterium mediated transformation efficiency varies with different soybean genotypes. Furthermore, greatly varying levels of reporter gene expression are observed after selfing and in various developmental stages in transgenic plants. Based on our present understanding of the gene silencing phenomena in transgenic plants and the fact that the soybean genome has a high content of heterochromatin, we propose that the varying transformation efficiencies and levels of gene expression in soybean may be due to the presence of a rich heterochromatin environment and high level of methylation in the genome. To test this hypothesis, we treated soybean seedlings with the DNA methylation inhibitor 5-azacytidine as well as the synthetic plant hormone 2-4-D before transformation with *A. rhizogenes*. We observed a significant increase in the GUS reporter gene expression in soybean root hairs and about 4.5% of GUS gene insert were identified by PCR from hair roots without GUS expression, supporting our hypothesis. Statistical variance analysis of GUS expression data showed the two agents reduced the genotypic differences to non-significant levels. Furthermore,

these two agents do not produce any detrimental effects on the soybean during development.

Keywords: soybean, *Agrobacterium* transformation, DNA demethylation, 5-azacytidine, transgenic roots.

Abbreviations: 5-aza, 5-azacytidine; 2-4-D, 2-4-dichlorophenoxyacetic acid; GUS. β-glucuronidase; Mtases, DNA methyltransferases; X-Gluc, 5-bromo 4-chloro 3 - indolyl β-D glucuronic acid; 35S, the promoter from cauliflower mosaic virus 35S gene

Introduction

Highly efficient *Agrobacterium* mediated transformation has been reported with both dicotyledonous and monocotyledonous species [1,2]. Furthermore, is possible to transfer more than 150 kb of DNA using *Agrobacterium* mediated plant transformation into a plant chromosomes [3]. And, with certain genotypes. it is possible to obtain a 37% rate of *Agrobacterium rhizogenes* mediated hairy roots on soybeans [4]. But, there is no efficient *Agrobacterium* mediated transformation procedure for most soybean varieties, especially the more useful agronomic varieties.

In gene silencing studies in transgenic plants, variations and depression of the transferred genes for expression correlates with DNA methylation [5,6]. The cytosine-5-methyltransferases inhibitor 5-azacytidine has been reported to increase expression of the GUS reporter gene as well as to re-activate gene expression in several different plants [7,8,9,10,11,12]. *Agrobacterium* mediated transformation of soybeans with the GUS reporter gene varies with different genotypes and environmental effects. We asked the question whether the low

Agrobacterium mediated transformation efficiency, and varying levels of transgenic gene expression, is due to the high percentage of methylated regions of the soybean genome. To obtain a partial answer to this question, we designed a *Agrobacterium* transformation preinoculation medium containing 5-azacytidine and 2-4-D. Our data suggest that the previously observed low level of GUS gene expression in transgenic plants may be correlated with the level of DNA methylation. Our overall objective was to improve the *Agrobacterium rhizogenes* mediated transformation level to test putative cloned disease resistance candidate genes that infect soybean roots.

Materials and methods

Plant materials and growth conditions

Two soybean varieties, Williams and Hark, were obtained from Dr. Walter Fehr, Agronomy Department, Iowa State University. Cultivar L85-3044 were obtained from the soybean seed center, Urbana, IL. All plants were grown in a computer managed greenhouse.

Agrobacterium rhizogenes culture for soybean transformation

A single clone of *Agrobacterium rhizogenes* strain K599 with a disarmed binary plasmid pBI35S GUS INT (obtained from G. Vancanneyt, Institüt für Genbilogische Forschung, Berlin) which contains a GUS gene with an intron, and a NPTII selection maker gene between the right and left border regions. The strain was grown in rich medium with 50 μg/ml of kanamycin to exponential phase. 100 μM of acetosyringone dissolved in 50% of ethyl alcohol was added to the liquid

culture just before pelleting the *Agrobacterium* culture. The supernatant was decanted and the *Agrobacterium* pellet was resuspended at an OD_{600} of 4.0 to 5.0 in sterile buffer. This resuspended culture was immediately used to inoculate 6-7 day old soybean seedlings germinated from a pre-inoculation culture medium.

Inoculation of soybean seedlings

The soybean inoculation procedure was based on the protocol of Savka *et al.*[4]. Soybean seeds were first sterilized by chloride gas; one layer of seeds was placed in Petri dishes in a desiccator with 100 ml of commercial Clorox containing 3 ml of concentrated HCL. The seeds were sterilized for about 16 hours and then germinated in the dark on pre-inoculation medium at 24°C for six to seven days. The pre-inoculation medium consisted of 1/10 Gamborg's B-5 medium (from Gibco Life Technologies, Inc. Grand Island, NY 14072 USA) with 8 g sucrose and 8 g of phytoagar per liter. After the autoclaved pre-inoculation medium had cooled, 100 μM of 5-azacytidine and 1 mg/l of 2-4-D were added. The soybean seedlings were inoculated with a sterile 1 ml syringe needle on the hypocotyl just beneath the cotyledon. Approximately 2 to 3 μl of fresh *Agrobacterium* culture was injected into 5 to 10 lightly wounded sites. After inoculation, soybean seedlings were transferred to a 10 x 7 x 7 cM autoclavable Magenta jars containing 25 to 30 ml of 1/10 Gamborg' B-5 medium with 30 g sucrose and 8 g of phytoagar per liter. 30 μg/ml of kanamycin was present in this medium. After about 7 to 15 days, roots beneath the inoculation sites were cut off, the inoculated soybean seedlings were transferred into a new Magenta jar containing 30 to 40 ml of liquid Mon Mor (MM) medium [4] with 100 μg/ml of kanamycin, 200 μg/ml carbenicillin, 50 μg/ml clavulanic acid, 100 μg/ml of cefotaxine and

amoxicillin. After 2 to 3 weeks of culture, the transgenic hairy roots were cut off for GUS staining and PCR analysis.

GUS assay

The newly grown roots were assayed for β-glucuronidase (GUS) activity by incubation in a 0.5 ml of 5-bromo 4-chloro 3 -indolyl β-D glucuronic acid (X-Gluc) for 12 hrs at 37°C [13]. The stained transgenic roots were de-stained and preserved in 75% ethanol. Each tube and plant was assigned an identification number to match the PCR results. Two universal primers from the conserved domain of the *vir*D2 gene of *Agrobacterium* were used to identify *Agrobacterium* residual contamination on transgenic roots [14] and were: upper universal primer (5'-ATG CCC GAT CGA GCT CAA GT-3'), antisense primer (5'-CCT GAC CCA AAC ATC TCG GCT GCC CA-3'). Two conserved sequences in the GUS gene were also used for identification, and were: 5'-TTC TTT AAC TAT GCC GGG ATC CA-3' and the antisense strand was 5'-CGA GTG AAG ATC CCC TTC TTG TT-3'.

DNA isolation and PCR conditions

Two rapid DNA isolation methods were used for PCR analysis of soybean DNA from the transgenic roots. Both methods produce repeatable results. The first methods was to randomly cut 1 mm long tissue from the selected transgenic roots and add 15 µl of GeneReleaser solution (GeneReleaser, Bio Ventures, Inc. 848 Scott Street Murfeesboro, TN 37129) in a 0.5 ml microcentrifuge tube. The specimen is overlaid with 80 µl of mineral oil, closed and cooked in a microwave (900 Watts) oven for 5 minutes. The sample is then immediately put in a preheated PCR machine for 7 minute at 80° C, then in ice or a PCR master mix

solution is added to start the PCR cycle. The second rapid DNA isolation methods was a modification of Klimyuk's protocol [15] as follow: a piece of pretreated tissue (less than 1 mm long) in 10 µl of water is boiled for 10 minutes. the PCR reaction component are then added to a total of 50 µl reaction volume and the reaction begun. The 2-3 mm long root tissue was treated by boiling in 50 µl of 0.25 M NaOH in a autoclaved Eppendorf tube at 100°C for 30 sec in a preheated themocycler. This was followed by neutralization with 50 µl 0.25 M HCL and 25 M Tris-HCL, pH 8.0, 0.25% Nonidet P-40 (Sigma). The treated tissue can be used immediately or kept at 4°C for at least a week. The PCR master mix consisted of 1 unit Taq polymerase, 5 pmoles of each primer, 1% (v/v) of dimethyl sulfoxide, 5 µl of 10X Mg-free thermo DNA polymerase buffer, 200 µM of each dNTP and 5 µl of 0.05 mM MgCl. The cycling parameters were as following: 94°C for 5 minutes, 57° C for 2 minutes, 72° C for two minutes, then 35 cycles of 94° C 20 second, 57°C for 30 second and 72°C for 2 minutes, followed by 72°C for 5 minutes. The amplified fragment for β-glucuronidase is about 1 kb and 338 bp for the *vir*D2 gene. The amplified DNA products were separated in 1.2% agarose in TAE buffer and stained with ethidium bromide. Photographs were taken via a video camera interfaced to a Macintosh computer.

Statistical data analysis

Due to the nature of the experiments, the data were analyzed by the Statistical Analysis System general linear model procedure[16]. The variance analysis of 5-azacytidine and 2-4-D effects on soybean flowering time was analyzed as randomized block designs. The variance analysis of GUS expression were analyzed as a nest model.

Results

Increase GUS expression *in vivo* by the combined effect of 5-azacytidine and 2-4-D

It has been reported that treatment of *Agrobacterium* on tobacco leaf disks with 5-azacytidine can increase transgenic gene expression 4 to 6 fold [17]. Treatment of callus with 5-azacytidine can also lead to a 3% to 96% increase in the regeneration of transformed shoots in *Arabidopsis thaliana* [7]. It is known that *Agrobacterium rhizogenes* T-DNA coded *rol* genes cause plant cells to become more sensitive to endogenous auxin [18,19]. And, it has also been reported that the phytohormone 2-4-D can increase the activity of *osc* elements, a family of related 20 bp DNA sequences that act as an enhancer element for a number of *Agrobacterium* genes as well as the 35S promoter of cauliflower mosaic virus [20,21]. To test if the DNA demethylation agent 5-azacytidine and plant synthetic hormone 2-4-D have an effect on transformation efficiency in soybean, we transformed germinating seedlings with an *A. rhizogenes* strain possessing the GUS reporter gene located between the left and right border regions of a transforming vector. GUS expression was tested in 468 putative transgenic roots that were treated with 3 different concentrations of 5-azacytidine and 2-4-D in the seed germination medium. The soybean seedlings were inoculated with *Agrobacterium rhizogenes* strain K599 which possessed the plasmid p35S GUS INT from *Agrobacterium tumefaciens*. In some experiments, this strain was also treated with 50 μM 5-azacytidine during its culture. GUS activity in the transformed plants was used as a measure of transgene expression efficiency. Table 1 shows the results of T-DNA mediated transformation efficiency in

soybean seedlings with various combinations of 5-azacytidine and 2-4-D. The data in Table 1 (treatment 16) indicate that GUS expression increased from 32% (control treatment) to 86% when treated with 50 μM 5-azacytidine and 1 mg/L of 2-4-D. The overall mean of GUS expression for various combinations of the 3 factors is summarized in part B of table 1. To determine which factors showed statistically significant effects on the GUS expression levels, we performed a variance analysis on the data using the general linear models procedure. The results presented in Table 2 show that variance source D(M) and M x R are not statistical significant. This is interpreted to mean that the effect of 2-4-D on GUS expression is not significant but the overall effects of the combination (T(MxD)) of 5-azacytidine with 2-4-D on GUS expression is highly statistically significant. The overall effect of 5-azacytidine (M) on GUS expression is also significant. The significant difference within the replication (R) means that each root within the treatment group expresses GUS significantly different because some roots show no GUS expression.

GUS expression in different soybean genotypes

Soybean genotypes vary in their susceptibility to *Agrobacterium* infection [4,22,23,24]. Our previous data. based on the GUS expression level in 10 soybean genotypes, was consistent with these findings (data not shown). If our hypothesis is correct with respect to methylation patterns in soybean genomes, the effect of preinoculation of 5-azacytidine and 2-4-D in germination medium should reduce any genotypic differences seen in the levels of GUS expression. As seen from the data presented in Table 3, the overall GUS expression efficiency of three soybean genotypes, treated with 5-azacytidine and/or 2-4-D shows no significant differences. Table 3 shows the variance analysis of the effects of preinoculation

on GUS expression from transgenic soybean roots using three different genotypes in a random block design. These three genotypes previously showed significant differences in transformation efficiency without preinoculation treatment. Again, the combined effect of treatment on GUS expression is highly significant.

Effects of 5-azacytidine and 2-4-D on flowering of soybean plants

It was reported that treatment with the DNA demethylating agent 5-azacytidine induced nonvernalized *Arabidopsis thaliana* plants to flower significantly early than untreated controls [25]. A similar phenomena was also reported in flax, *Linum usitatissimum L,* [26]. Five-azacytidine was also reported to induce both dwarfism and DNA demethylation in maize [27]. And, hypomethylation mutants in *Arabidopsis thaliana* show a spectrum of morphological abnormalities including altered flowering times and flower structure [28]. Thus, it is very possible that the combined effects of 5-azacytidine and 2-4-D could cause abnormalities in soybean development. We had observed that treated seedling showed morphological changes, such as root enlargement and the inhibition of root hair growth (Figure 1). An enlargement of root tissue can be seen in seedlings germinated with 2-4-D as well as a reduction in the number of root hairs when germinated in the presence of 5-azacytidine. A callus-like structure is seen at root tips of seedling germinated in the presence of both 5-azacytidine and 2-4-D. To ascertain if mature plants also possess morphological and developmental differences, we planted 84 pre-treated seedlings in the greenhouse. We found that all treated plants had normal seed set and did not show any morphological differences from the control plants. An analysis of the data is presented in Table 4, and shows no significant difference among the treated plants vs. control plants with respect to flowering time. There is also no

significant difference in flowering time for replications within each treatment. These data suggest that the combined effects of 5-azacytidine and 2-4-D did not have any long term detrimental effects on the soybean development.

PCR verification of transformation

The plasmid used in our transformation experiments is a disarmed binary vector in *Agrobacterium rhizogenes* strain K599. This plasmid has a p35S promoter before the GUS reporter gene and possesses an intron within it. Thus, the GUS gene should not be expressed unless integrated into a plant genome. Due to the paucity of root tissue, it was difficult to obtain sufficient material for Southern analysis, thus we used the PCR technique to rule out potential false positives due to expression of the GUS gene in the absence of integration into root tissue. The *virD2* gene possesses a highly conserved domain and was used to design a pair of PCR primers to detect the presence of *Agrobacterium* [14,29]. The pGUS INT plasmid does not possess a *virD2* gene within the left and right border region of T-DNA, but the helper plasmid does, thus the PCR primers will detect the presence of *A. rhizogenes*, but not the integrated sequences. In addition, two primers were used to amplify the β-glucuronidase gene to verify the presence of an integrated sequence. Figure 2 shows the PCR analysis of transgenic soybean roots. Lane 1 is 1 kb molecular size marker; lane 2 is the 338 bp long *vir*D gene PCR product from a single clone of *Agrobacterium rhizogenes* strain K599; lane 3-5 are the PCR products of two conserved domains from the GUS gene; lane 3 is from a root without GUS expression; lane 4 is from a root which show GUS expression; and lane 5 is from non-transformed soybean genomic DNA. All PCR analyses from random sample of GUS expression roots showed no contamination by *Agrobacterium*. However, GUS insert fragments were identified by PCR

analysis with primers for GUS gene and VirD gene from 5 out of 110 random selected non or weak-GUS expression roots (about 2 years old GUS staining samples in 75% of ethonal). We counted the roots contaminated by residual *Agrobacterium* if both GUS gene and VirD gene products present in the same roots. This PCR analysis results indicated there was at least 4.5% of GUS gene inserts being methylated in our transgenic soybean roots. We confirmed 4.6% of possible GUS gene being methylated in transgenic soybean roots in a separate experiments .

Discussion

Variable *Agrobacterium rhizogenes* mediated transformation of soybean may due to genomic DNA methylation.

Methylated cytosine of DNA is present in all plants analyzed, and varies from 20 to 30% of all cytosine. Furthermore, the level of methylation of upstream gene sequences is inversely correlated with the level of expression [9,30] and methylation appears to be one of mechanism of inactivation of transgenes. In addition, *Agrobacterium* itself is methylated at about 0.5% of all cytosine, and transgenes may be already methylated before insertion into plant genomes. Soybean genotypes are highly variable with respect to their susceptibility to *Agrobacterium* infection. Efforts to understand the genetic basis of this variable susceptibility to the *Agrobacterium* infection [22;31] have shown that between two to three genes control the level of susceptibility. In contrast, our data suggest that different genotypes do not show significant differences in the level of susceptibility of *Agrobacterium* infection, but only after treatment of soybean seedlings with 5-azacytidine and 2-4-D. The combined effects of these two agent

significantly increases the efficiency of transfer of the GUS gene from a maximum of 32% in the most susceptible soybean cultivar to 86% overall. There are at least 4.5% of GUS gene fragment present in soybean roots from *Agrobacterium* mediated transformation experiment that shows no GUS expression. These data suggest that DNA methylation may play an important role in GUS expression efficiency during *Agrobacterium* mediated transformation of soybean tissue. These findings are further supported by the report that 5-azacytidine can increase GUS activity in transgenic carrot callus [8], in tobacco [9] and in rice cell lines [10]. Furthermore, 5-azacytidine treatment led to an increase from 3% to 96% in the regeneration of transformed shoots in *Arabidopsis thaliana* [7]. Treatment with 5-azacytidine can also reactivate transgenes in tobacco [11] and petunia [12].

The study gene silencing in transgenic plants has revealed that the copy number as well as the position of the transgene is correlated with the expression level. And, this position effect is correlated with the amount of methylated adjacent chromosome region [5,8,30]. Gene silencing also correlates with the plant growth environment, for example, a transgenic tobacco line carrying the mutant *Arabidopsis thaliana* acetohydroxy acid synthase gene, *csr1-1*, in field grown plants is more methylated than greenhouse grown plants [32]. We propose that *Agrobacterium* mediated transformation (or infection) variation in soybean can be explained similarly to gene silencing, which is likely due to variable methylation. It was known that soybean (2n = 40) is a stable tetraploid with diploidized genomes [33] with 60% of repeated sequence scattered among single copy region [34,35]. Soybean pachytene chromosomes indicate that over 35% of the genome consists of heterochromatin; the short arms of six of the 20 bivalents are completely heterochromatic [36]. In fact, the soybean genome has a uniquely

high content of heterochromatin in the genome, and we interpret our findings to suggest that the low transformation and expression efficiency in *Agrobacterium* mediated transformation of soybean is due to rich heterochromatic regions of the soybean genome. Insertion in heterochromatic regions results in methylation of the newly inserted DNA, preventing its expression.

How to increase GUS expression efficiency in transformed plants

Efforts have been made to increase the transformation efficiency by construction of improved vectors. For example, retrotransposon derivative transformation booster sequence [37], or MARs/SARs have been incorporated into the transformation vectors to increase the efficiency of transformation in plants [38]. MARs/SARs are A/T rich DNA sequence organized in loop domains through interaction with a proteinaceous structure that binds to the nuclear matrix or scaffold *in vitro* [39]. However, MARs/SARs are less effective in *Agrobacterium* mediated transformation than direct DNA mediated transformation such as gene bombardment [38]. Since soybean has such a high percentage of heterochromatin in its genome and the main functions of MARs/SARs is to protect the inserted gene from methylation by its adjacent methylated chromosome regions, we do not believe that the increasing soybean transformation efficiency we have observed is due to this reason.

The factors that influence *Agrobacterium*-mediated transient expression of *gusA* in rice have been investigated [40]. They showed that 2-4-D at 2 mg/L inhibited GUS transgene expression if present only during seed germination, but not at 0.5 mg/L during germination and 6 mg/L 2-4-D during cocultivation of the explants with *Agrobacterium tumefaciens* enhanced GUS transgene expression. Our data

suggest that 2-4-D alone does not increase the level of GUS transgene expression and only has an indirect effect when it was combined with 5-azacytidine. 2-4-D may play a role of loosening cell wall structures, and thus create a more favorable environment for *Agrobacterium* infection. The classical model suggests that auxin-induced proton extrusion lowers the pH of the cell wall thus activating cell wall enzymes that cleave load-bearing bonds in cell wall [41].

The combined effects of 5-azacytidine and 2-4-D showed that they did not have any detrimental effects on soybean during development. These findings, therefore, may assist in developing an efficient *Agrobacterium* mediated transformation system in soybean.

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Table 1. The average level of GUS expression in soybean transgenic roots treated with 5-azacytidine and 2-4-D during seeds germination or growth of *Agrobacterium*

Part A: Mean of GUS expression by treatment

Trt No.	5-aza (μM)	2-4-D (mg/L)	5-aza (vector)	N	Mean (%)	SD
1	0	0	yes	26	0.60	0.25
2	0	0	no	28	0.32	0.25
3	50	1	yes	21	0.49	0.30
4	50	1	no	23	0.54	0.35
5	100	2	yes	31	0.34	0.32
6	100	2	no	18	0.44	0.23
7	0	0	yes	36	0.44	0.25
8	0	0	no	27	0.58	0.34
9	50	1	yes	24	0.72	0.24
10	50	1	no	26	0.61	0.27
11	100	2	yes	18	0.62	0.36
12	100	2	no	28	0.69	0.33
13	0	0	yes	28	0.48	0.33
14	0	0	no	28	0.57	0.28
15	50	1	yes	26	0.36	0.22
16	50	1	no	28	0.86	0.16
17	100	2	yes	28	0.60	0.38
18	100	2	no	24	0.71	0.25

Part B: Overall mean of GUS expression

5-aza (μM)	N	Mean	SD
0	147	0.50	0.30
50	159	0.59	0.31
100	162	0.60	0.32

2-4-D (mg/L)	N	Mean	SD
0	173	0.50	0.29
1	148	0.60	0.31
2	147	0.56	0.34

Vector/5-aza	N	Mean	SD
yes	238	0.51	0.32
no	230	0.60	0.31

Trt=treatment combination; N=number of roots tested; SD=standard diviation; Vector/5-aza = if *Agrobacterium rhizogens* was treated with 50 μM 5-azacytidine during its culture;

Table 2. Variance analysis of GUS expression from transgenic soybean roots that were treated with combinations of 5-azacytidine and 2-4-D during seed germination

Source	df	SS	F-Value
M	2	1.74	9.9 **
D(M)	6	1.33	0.37 ns
T(M x D)	9	5.29	6.68 **
R	34	2.36	0.79 **
M x R	58	3.88	0.76 ns
Error	358	31.45	
Total	467	47.17	

M=5-aza; D=2-4-D; T=if T-DNA was treated with 5-aza; R=replication; **= highly significant at P≤0.01; ns= not significant; df=degree of freedom; SS=type III sum of squares;

Table 3. Variance analysis of GUS expression from three genotypes* of transgenic soybean roots treated with combinations of 5-azacytidine and 2-4-D during seed germination

Source	df	SS	F-Value	(P > F)
Genotype (G)	2	0.21	0.17 ns	(0.845)
Treatment (G)	12	7.23	28.8 **	(0.000)
Replications (R)	41	7.88	9.18 **	(0.000)
G x R	54	0.65	0.58 ns	
Error	207	4.33		
Total	316	19.73		

* Hark, Williams, and L85-3044; **= highly significant at P≤0.01; ns= not significant; df=degree of freedom; SS=type III sum of squares;

Table 4. Variance analysis of flowering times of soybean plants that were treated with 4 combinations of 5-azacytidine and 2.4.D during the seeds germination in a random block design experiment

Source	df	SS	F-Value
Replications (R)	6	1.9	0.25 ns
Treatment (T)	3	8.14	2.17 ns
R x T	18	7.52	0.33 ns
Error	56	70	
Total	83	87.57	

**= highly significant at P≤0.01; ns= not significant; df=degree of freedom; SS=sum of square

FIGURE LEGENDS

Figure 1. Root morphological changes after seeds were germinated on pre-innoculation germination medium. An enlargement of root tissue can be seen on the seedlings from germinaton medium containing 2-4-D, and a reduction in the number of root hairs is seen in seedlings germinated in the presence of 5-azacytidine. A callus-like appearance of tissue is seen at root tips of seedling germinated in the presence of both 5-azacytidine and 2-4-D.

Fig 2. PCR analysis of transgenic soybean roots. Lane 1 is 1 kb molecular size marker; lane 2 is the 338 bp long *vir*D gene PCR product from a single clone of *Agrobactreium rhizogenes* strain K599; lane 3-5 are the PCR products of conserved domains from the GUS gene; lane 3 is from a root without GUS expression; lane 4 is from a root which showed GUS expression; lane 5 is the control using non-transformed soybean genomic DNA.

Figure 1

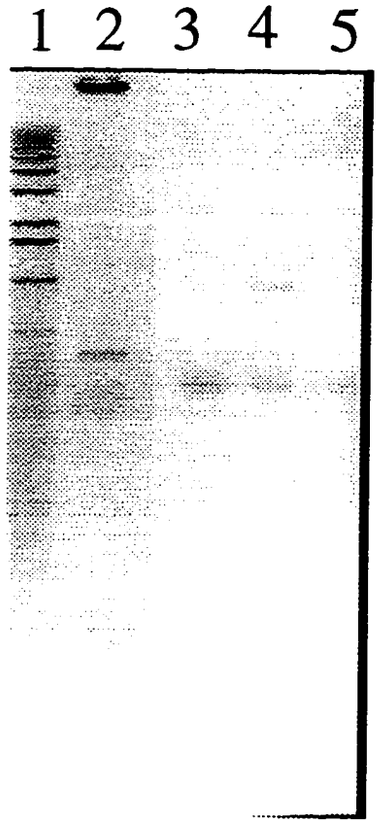

Figure 2

CHAPTER V: A PROTOCOL FOR SCREENING RESISTANCE TO *PHYTOPHTHORA SOJAE* IN *AGROBACTERIUM RHIZOGENES* MEDIATED TRANSGENIC SOYBEAN ROOTS

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Abstract

To verify the cloning of any *Phytophthora* plant resistance gene, a relatively simple and rapid screening protocol is necessary. Transformation of soybean is presently difficult and time consuming. Herein we describe a procedure for assaying for the presence of putative resistance gene clones using an *in vitro* transgenic root coloration reaction. This procedure can be used for both screening gene expression in transgenic roots as well as roots from an F_2 population in soybean. This protocol utilizes an *in vitro* color reaction of transformed roots resulting from an *Agrobacterium rhizogenes* infection as well as a thin-layer chromatography analyses of the carbohydrates found in infected and uninfected roots of susceptible and resistant lines of soybean.

Phytophthora root and stem rot of soybean is one of the most destructive fungal diseases in most of the soybean-producing areas of the USA and other countries. It was estimated that about $188 million was lost per year in the period 1988-1991 due to this disease (Doupnik, 1993; Schmitthenner, 1989; Athow, 1987). Infections by *Phytophthora sojae* can result in complete yield loss

in susceptible soybean varieties in some areas (Yang, et al., 1996). This disease has been combated by the introduction of resistance alleles into agronomically important cultivars, but to broaden the soybean breeding program, it would be useful to obtain clones of the *Phytophthora* resistance alleles from soybean. To attain this objective we have developed a screening protocol for testing putative *Phytophthora* resistance gene clones from soybean. Screening of putative clones can also be done using soybean transformation and regeneration, however, current transformation systems are tedious and inefficient. We have adapted the *Agrobacterium rhizogenes* mediated transformation protocol of Savka et al. (1990) for screening *Phytophthora* resistance in soybean. The following coloration assay can be used for both screening resistance alleles as well as screening F_2 individuals in a cross.

The conventional method for screening *Phytophthora* resistance in soybean is to insert a small segment of mycelium into an incision site in the hypocotyl of soybean seedlings of about two weeks old. However, this inoculation method, as well as the zoospores inoculation method, will eventually kill sensitive seedlings (Eye et al., 1978). This can be a problem when screening individual soybean seedlings when the genotype has not been identified and the plant is needed for future testing. The procedure described in this manuscript allows testing for resistance and survival of the tested plant. It can also be useful in analysis of bulked segregant or pooled F_2 populations when closely linked polymorphic markers need identification (Michelmore, et al., 1991; Churchill, et al., 1993; Tanksley, et al., 1995). Heterozygous and homozygous resistance progeny can be distinguished by segregation within $F_{3:4}$ individual lines. Thus, an inoculation method for screening F_2 populations is necessary to obtain information about phenotypic segregation (3:1) as well as harvesting F_3 seeds. This can save one

generation of time when trying to identify closely linked polymorphic markers using two bulked segregate or pooled F_2 populations.

Assay for *Phytophthora* resistance in soybean

Smith, et al. (1991) reported an *in vitro* assay for evaluation of *Phytophthora* resistance using soybean taproots, but this method is difficult to use for screening F_2 populations as it is very time consuming and expensive. Thus, we developed an *in vitro* screening method. This procedure involves use of cultured soybean roots and inoculated with a spore suspension of *Phytophthora*. The seeds are first sterilized by chlorine gas; one layer of seeds is placed in a Petri dish in a desiccator with 100 ml of Clorox containing 3 ml of concentrated HCl. The seeds are sterilized for about 16 hours and then germinated in the dark on a medium containing 1/10 Gamborg's B-5 medium with 7 g of phytoagar (Gibco Scientific) per liter at 24°C for seven to ten days. *Phytophthora* race 5 and 25 were isolated and identified from soybean fields in Iowa (Yang, et al., 1996). The zoospore suspension was obtained according to Eye's method (Eye, et al., 1978). The inoculation of *Phytophthora* was done by the sterile removal of the roots from germinating soybean seedlings and placing them on *Phytophthora* growth medium consisting of 1/10 Gamborg's B-5 medium with 7 g of oatmeal agar (Sigma Cat No. 0-3506) per liter. This methods was first used to test known *Phytophthora* resistant and sensitive soybean varieties. We tested resistant variety L85-3044, and sensitive variety Williamss with *Phytophthora* *Phytophthora* race 5. Figure 1 shows two roots; one from a resistant line L85-3044 which remained colorless, and one from the sensitive line Williamss, which develops a brown coloration after about 30 hour of infection. As a control, the traditional hypocotyl inoculation method was also used and verified the genotype

of the seeds used in the color reaction assay as well as the pathogenicity of isolates.

To further study the reliability of this color reaction assay, we developed an F_2 population by crossing near isogenic lines L85-3044 and Williams. The F_1 seeds were first verified by plant isozyme pattern analysis (data not shown). F_1 seeds were then grow in the greenhouse to yield an F_2 population of seeds. F_2 seeds were then bulked from individual $F_{1:2}$ plants, sterilized and germinated. About 2-3 cM of the tap roots was removed from each seedling and inoculated with *Phytophthora* zoospore suspension solution and cultured for one to two days. Table 1 is the chi-square test for the expected 3:1 ratio of diseased (color) and healthy (colorless) roots in four trials. The analysis of 395 roots of F_2 plants show that 286 were resistant and 109 susceptible. Chi-square analysis showed a 3:1 ratio was accepted. F_2 analyses showed a direct relationship between the color reaction and predicted genotype. In contrast, the conventional hypocotyl inoculation method results in the loss of the homozygous recessive genotypes. Figure 2 shows the use the traditional hypocotyl inoculation method for screening F_2-derived lines. Homozygous resistant lines grow and about 1/3 of heterozygous plants die. All plants died from the homozygous recessive lines.

This procedure was slightly modified for use in testing *Agrobacterium* mediated transgenic roots. The individual transgenic roots were cut from *Agrobacterium* inoculation sites and washed three times with sterilized water. The root were then placed on an 7% oatmeal agar plate grown with *Phytophthora* mycelium. Changes in coloration were recorded over a period of two or three days. The coloration was recorded as 1 for resistant with no coloration, 2 for medium coloration and 3 for complete coloration (Table 1). A variance analysis was also conducted on the data using the Statistical Analysis System general linear model procedure (SAS, 1988).

Transformation of soybean roots was done using *Agrobacterium rhizogenes* using a modification of the protocol described by Savka, et al. (1990). Briefly, a single clone of *Agrobacterium rhizogenes* strain was used. This strain possessed a disarmed binary plasmid p35 GUS INT (obtained from G. Vancanneyt, Institüt für Genbilogische Forschung, Berlin) which has an intron-containing GUS gene, and a NPTII selection maker gene between the right and left border regions. The strain was grown in rich medium with 50 μg/ml of kanamycin and immediately used to inoculate 6-7 day old soybean seedlings germinated from a pre-inoculation culture medium. After 2 weeks of culture, the transgenic root hairs were removed and put into a root growth medium with antibiotics which kill residual *Agrobacterium*. After 2 weeks of culture, the transgenic root hairs were removed for GUS staining, PCR analysis and screening for *Phytophthora sojae* resistance.

For transformation of other genes into soybean roots, the GUS gene was removed from plasmid p35 GUS INT by restriction enzyme digestion with *Bam*H1 and *Sst*1, and replaced with various gene candidates. The constructed plasmids were transformed into *Agrobacterium rhizogenes* strain K599. Transgenic roots from candidate genes were verified using PCR (the upstream primer was from the 35S promoter region and the downstream primer was from the candidate gene). Soybean transgenic root hairs from control plants inoculated with plasmid p35S GUS INT were identified by PCR analysis and β-glucuronidase (GUS) activity by incubation in a 0.5 ml of 5-bromo 4-chloro 3 - indolyl β-D glucuronic acid (X-Gluc) for 12 hrs at 37°C (Jefferson, 1987). The stained transgenic roots were de-stained and preserved in 75% ethanol. Each plant was assigned an identification number and matched to the PCR and *Phytophthora* screening data. For PCR analysis, a rapid DNA isolation procedure was used. In this procedure, randomly cut 1 mm long tissue from the selected

transgenic roots was combined with 15 µl of GeneReleaser solution (GeneReleaser, Bio Ventures, Inc. 848 Scott Street Murfreesboro, TN 37129) in a 0.5 ml microcentrifuge tube. The specimen was overlaid with 80 µl of mineral oil, closed and cooked it in microwave oven (900 Watts) for 5 minutes. The sample was immediately put in a preheated PCR machine for 7 minute at 80° C. The sample tube is then put in ice, or PCR master mix solution was added to start the PCR reaction. The PCR master mix consisted of 1 unit *Taq* polymerase, 5 pmoles of each primer, 1% (v/v) of dimethyl sulfoxide, 5 µl of 10X Mg-free thermophilic DNA polymerase, buffer, 200 µM of each dNTP and 5 µl of 0.05 mM MgCl in a total volume of 50 µl. The cycling parameters were as following: 94°C for 5 minutes, 57°C for 2 minutes, 72°C for two minutes, then 35 cycles of 94°C 30 second, 57°C for 30 second and 72°C for 2 minutes followed by 72°C for 5 minutes. Two conserved sequences in GUS gene were also used for identification of transgenic control plants, and were: 5'-TTC TTT AAC TAT GCC GGG ATC CA-3' and the antisense strand 5'-CGA GTG AAG ATC CCC TTC TTG TT-3'. The amplified fragment for β-glucuronidase is about 1 kb. The amplified DNA products were then separated in 1.5% agarose with TAE buffer and stained with ethidium bromide.

Three soybean varieties were used for transformation and disease resistance screening: *Phytophthora* resistant line L85-3044 and susceptible lines Williams and Hark. About 40% of transgenic roots were obtained by above *Agrobacterium rhizogenes* mediated transformation procedure. About 159 transgenic roots were used in color assay for screening *Phytophthora sojae* resistance. Table 2 is an analysis of variance for the coloration assay from an soybean transformation experiment. The study was designed to investigate the effects of three soybean varieties (Williams, L85, Hark), three plasmid vectors

(pc06, pt5-19 and control plasmid p35S GUS INT) and two pathogen races (race 5 and race 25) on the transgenic roots. Table 2 shows the results of variance analysis of coloration assay from plants varieties that were transformed with three separate plasmid constructs for the color assay. The effect of plasmid construct on the color assay is found to be highly significant. On the other hand, the effects of variety and pathogen race on the coloration assay were not significant. In this experiment, *Phytophthora* resistant variety L85 was transformed with the same plasmid as the positive control. As we expected, transgenic roots from the resistant variety L85 produced susceptible color reactions after *Phytophthora* infection. This result may be due to possible gene co-suppression in transgenic plants (reviewed by Matzke, and Matzke, 1995; Finnegan, and McElory, 1994). In our previous experiments, we also observed that resistant and susceptible soybean varieties inoculated with either race 5 or race 25 produced consistent results. The variance with respect to race (Table 2) is thus not significant. From the data presented in Table 1 and Table 2, we conclude that the root color assay is a reliable method for screening *Phytophthora* resistance in soybean.

Carbohydrate production

In cultured transgenic roots more than four weeks old, we have consistently observed that when the media contains a 2% to 3% sucrose concentration, roots do not respond to*Phytophthora* infection, even when using various inoculation methods. In this respect, it has been reported that immature trifoliolate leaves from plants fed various sugars or glyceollin promote resistance to *Phytophthora sojae* race 1 in both soybean Harosoy 63 and susceptible cultivar Harosoy (Ward, 1989). Also, general defense-related plants proteins, such as chitinases and ribosome-inactivating protein trichosanthin, accumulate after *Agrobacterium*

mediated transformation (Savary and Flores, 1994). As a consequence, we always score disease resistance in our assay by comparing transgenic roots with roots obtained by infection with *Agrobacterium* containing a disarmed binary plasmid p35 GUS INT.

In the early stages of the infection of soybeans by the fungal pathogen *Phytophthora,* soybean extracellular β -1, 3-endoglucanase attack the invading fungus releasing elicitor active carbohydrates from the *Phytophthora* cell wall (reviewed by Keen and Dawson, 1992; Benhamou, 1996). The released β-glucan elicitors can bind to receptor proteins which have significant homology to glucanases (Umemoto, et al., 1997). This leads to a host defense response, including phytoalexin and polyphenol production and leads to coloration of the infected roots. The endoglucanase not only release fungal wall elicitors (which have four different backbone chains of varying length and side branches) but also glucose and other sugar molecules (Okinaka et al., 1995). It has recently been reported that glucose as well as other sugars may play an important role in the mediating signal transduction in plants (reviewed by Smeekens and Rook, 1997). Sucrose enhances the expression of wound inducible potato inhibitor genes responsible for plant defensive chemicals to protect against herbivores. In addition, digestibility and nutritional quality of the leaf protein is decreased (Johnson and Ryan, 1990). As a consequence, we have investigated how carbohydrates products from two soybean near isogenic lines differ after infection by *Phytophthora.*

Thin layer chromatography (TLC) was used for analyses of carbohydrates (White and Robyt, 1988) from soybean roots. The soybean roots tissue, with or without *Phytophthora* infection, was carefully weighted and homogenized with 0.5 mg/ml of 0.02% sodium azide in a 1 ml Eppendorf tube. This tube was centrifuged for 3 minutes at 3000 x g. The upper solution was transferred to a

new 0.5 ml Eppendorf tube, heated at 80°C for 15 minutes, and centrifuged 5 minutes. The upper solution was transferred into another 0.5 ml tube and used immediately, or could be stored at 4°C for months. A 10 x 10 cm Whatman K5 silica gel TLC plate (Sigma Chemical Co, St. Louis, MO) was used. About 1" above the bottom edge of the plate, samples were positioned 1.1 cM apart using a capillary glass tube. About 6 ul of samples were added on the TLC plate which was then placed into a chromatography jar containing the ascending buffer (85 : 15 v/v acetonitrile:water) 0.5 cM above the bottom. During elution, the lid was sealed with vacuum grease to prevent air circulation. The plate was intermittently dried four times (in an oven at 50°C for 8 to 10 minutes) between ascents. After the last ascent and drying, the plate was dipped into a revealing solution (a methanol solution consisting of 0.3% w/v N-1-(naphthyl) ethylenediamine and 5% v/v of concentrated sulfuric acid). The plate was then heated in an oven at 100°C for 10 min. For identification of the sample, the location of each sample spot was compared with standards.

Figure 2 shows the TLC analysis of roots tissue from uninfected and infected soybean. Line 1 is the mixtures of oligosaccharides TLC standards containing 2 mg/mL each of D-xylose, D-fructose, D-glucose, D-galactose, sucrose, cellobiose, melezitose, melibiose, and raffinose (from top to bottom). Line 2, 4 and 6 are L85-3044. Line 3, 5, 7 are Williams. Samples from roots of the cultivar L85 all possessed an extra band at the position of sucrose. To determine the monosaccharide composition of the oligosaccharide band, the band was acid hydrolyzed and three commercial available enzymes; α-glucosidase (Type 1), β-glucosidase (Type II), and invertase (Grade VII) were used in TLC analysis of root tissue samples from soybean. The result showed that this band (obtained from *Phytophthora* sensitive cultivar Williams) was not present, or present in trace amouts, in the resistant line (L85-3044) as sucrose. To perform the statistical

analysis of the TLC data, each TLC plates was video analyzed via a CCD video camera that was interfaced to a computer. After calibrating the image to a base line, each TLC band was plotted using NIH image software. Each TLC band was thus translated to a digital value, and then analyzed statistically. However, the variance analysis for overall carbohydrates products and for the three carbohydrates product (C1, C2, and C3) showed that the effects of variety, race and plasmid construct were not significant in the variance analysis. A multivariance analysis of the color assay with TCL data and others was thus conducted. The correlation of color assay with the TLC data and other data is presented in Table 3. To minimize possible artificial variations among individual lanes in each TLC plate, band 1(C1) and band 2 (C2) were used as an internal control. Thus, the value for each band was divided by the value of its arbitrary internal control. Table 3 show the correlation data (in the left lower side) are correlation value from the converted data, and the upper right data are the correlation values for the original image data. Correlation coefficients for the value between coloration assay and carbohydrate band 3 (C3) were about -0.1. The results presented in Figure 2 also indicate that the higher the color reaction the lower the value of TLC band 3 (C3). The variance analysis of TLC data and the probability values in the correlation coefficient matrix in Table 2 suggest that the TLC data can only serve as a reference data for screening *Phytophthora* resistance in transgenic roots.

There are at least 39 races of *Phytophthora* that have been reported in soybean (Henry and Kirkpatrick, 1995; Bhat and Schmitthenner, 1995). The above color assay and TLC method may help to screen *Phytophthora* resistance with other races of *Phytophthora* other than the races tested. The goal of obtaining an assay for *Phytophthora* as been realized in these studies and can be used in the cloning of resistance genes.

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Table 1. Chi-square test of diseased and healthy roots in four trials

Population Id	Total roots	Observed Values		Expected Valuse		K^2 Value
		Color	Colorless	Color	Colorless	
2-1-1	85	64	21	63.75	21.25	0.004
7-1-2	69	48	21	51.75	17.25	0.816
5-1-1	97	68	29	72.75	24.25	0.993
4-1-7	144	106	38	108.0	36.0	0.083
Total Sum	395	286	109	296.25	98.75	1.284=K^2T 1.896=K^2s

$X^2d = K^2s - K^2T = 0.612$; Tabular $K^2 = 13.28$ at 1% level; The 3:1 ratio is accepted.

Table 2. Variance analysis of coloration assay from transgenic soybean roots that were transformed with three plasmid vectors and innoculated with two *Phytophthora sojae* races

Source	df	SS	F-Value
Var(V)	2	1.92	1.83 ns
Vct(V)	6	26.13	8.26 **
Race(R)	1	0.27	0.51 ns
R* V	2	0.91	0.87 ns
R*Vct(V)	3	0.27	0.17 ns
Error	144	75.79	
Total	158	105.29	

V=varieties for L85, Williams and Hark; Vct=plasmid vector for pc6, pt5-19 and control plasmid vector p35S GUS INT; R=race 5 & 25; **= highly significant at P≤0.01; ns= not significant; df=degree of freedom; SS=type III sum of squares.

Table 3. The Pearson correlation coefficients analysis of coloration assay with soybean varieties inoculation with *Phytophthora* soja races and data from thin layer chromatography (TLC) analysis of carbohydrates

	Color	Var	Race	C1	C2	C3
Color	**1.0**	0.004	0.024	-0.084	-0.009	-0.110
	0.0	0.97	0.862	0.553	0.949	0.438
Var	0.119	**1.0**	-0.246	0.106	0.191	-0.091
	0.932	0.0	0.081	0.458	0.177	0.523
Race	0.019	-0.259	**1.0**	0.122	-0.036	0.143
	0.888	0.061	0.0	0.392	0.800	0.315
C1	0.121	-0.096	-0.038	**1.0**	0.152	0.075
	0.387	0.491	0.786	0.0	0.284	0.598
C2	0.063	0.072	0.039	-0.779	**1.0**	0.266
	0.649	0.606	0.779	0.000	0.0	0.058
C3	-0.103	0.030	0.177	-0.206	0.190	**1.0**
	0.462	0.827	0.203	0.137	0.171	0.0

The value of Pearson correlation coefficients in the left triangle of the matrix are for the converted carbohydrate TLC data. The right triangle in the matrix are the Pearson correlation coefficients values for the unconverted TLC data. Color = values from coloration assay; Var = varieties; C1 is the first (top) band in TLC plate, C2 and C3 are the second and the third major band in the TLC analysis.

FIGURE LEGENDS

Figure 1A shows the dead susceptible (genotype aa) plants that were innoculated by the conventinal methods; 1B two roots; one from a resistant line L85-3044 which remained colorless, and one from the susceptible line Williams, which shows brown coloration after about 30 hour of infection.

Figure 2 shows the Thin layer chromatography (TLC) analysis of roots tissue from uninfected and infected soybean. Samples from roots of the cultivar L85 all possessed an extra band at the position of sucrose. Line 1 is the mixtures of oligosaccharides TLC standards containing 2 mg/mL each of D-xylose, D-fructose, D-glucose, D-galactose, sucrose, cellobiose, melezitose, melibiose, and raffinose (from top to bottom bands). Line 2, 4 and 6 are L85-3044. Line 3, 5, 7 are Williams.

Figure 1

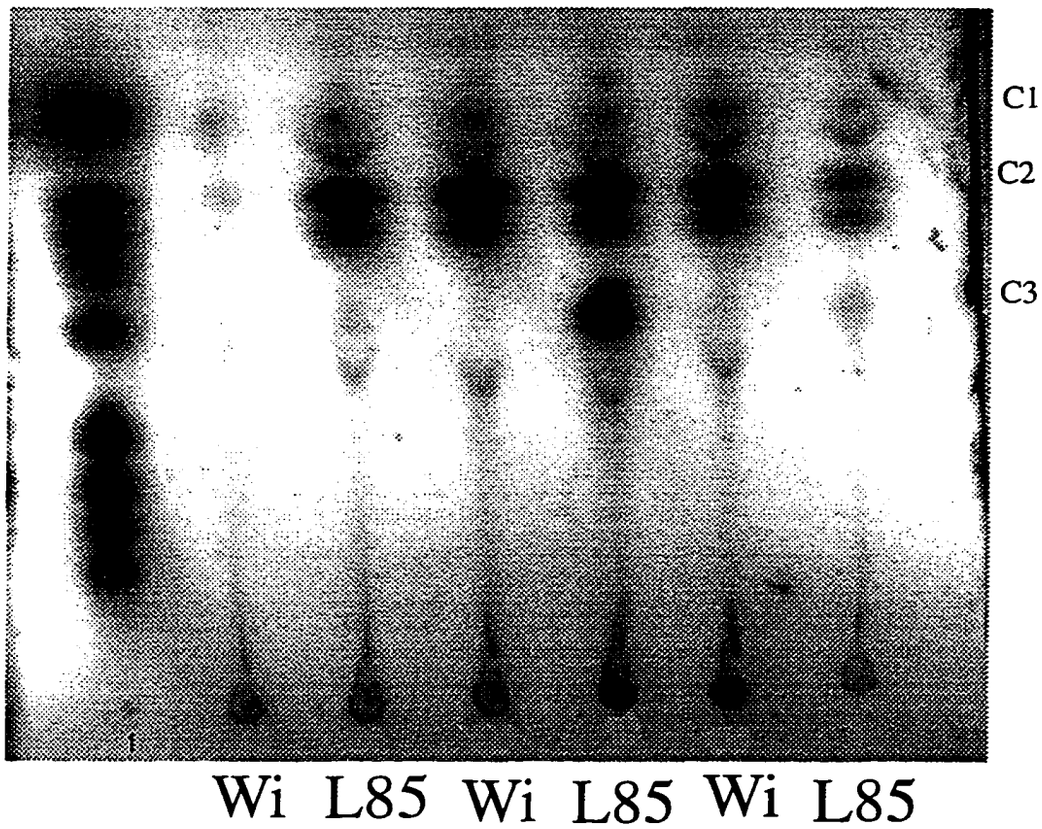

Figure 2

CHAPTER VI. GENERAL CONCLUSIONS

Phytophthora root and stem rot of soybean is one of the most destructive fungal disease in soybean-producing areas of the USA and other countries, and it can result in a complete yield loss in some areas. Cloning the *Phytophthora sojae* resistance genes from soybean may eventually help soybean breeders to fight this diseases either by molecular marker assisted backcrossing programs, or by the design of new fungicides deduced from understanding the mechanism of the interaction between the plant pathogens and host. To clone the resistance gene we have designed primers from conserved motifs in plant disease resistance genesand sued them in a modified differential display. We believed that this type of new cloning strategy will be very helpful in cloning genes from plant species with complex genomes.

Cloning and characterization of a yeast SNF2 homolog gene gmSNF2 for *Phytophthora* resistant

Sequence analysis of cDNA clone of gmSNF2 from soybean *Phytophthora sojae* resistant line L85-3044 showed that it has ca 45% homology with the yeast SNF2 gene. The putative protein of gmSNF2 has LRRs and leucine zipper motifs and a possible ATP binding domain. The yeast complementation test indicated that gmSNF2 has SNF2 function in the yeast SNF2 mutant. In addition, screening for *Phytophthora sojae* resistance with transgenic hair roots indicated that gmSNF2 gene could cause *Phytophthora sojae* resistance reactions in susceptible soybean lines. The SNF2 gene of yeast is a member of a large group of related proteins that exhibit a broad range of biological functions, such as gene-specific transcriptional activation, destabilization of reconstituted nucleosomes and transcriptional repression. The budding yeast preferentially

utilizes glucose for ATP production. Number of genes, including SUC2, which involved in carbon metabolism, are repressed when sufficient levels of glucose are present in the environment. This is known as carbon catabolite repression. It was reported that in the early stages of the infection of soybeans by the fungal pathogen *Phytophthora,* soybean extracellular β -1, 3-endoglucanases attack the invading fungus releasing elicitor active carbohydrates from the *Phytophthora* cell wall. Glucanases in soybean not only releases elicitors that have four different backbone chains of varying length and side branches but also glucose as well as other sugars from the fungal wall. It has been recently reported that glucose as well as other sugars may play an important role in the mediating signal transduction in plants. For example, various sugars or glyceollin promote resistance to *Phytophthora sojae* race 1 in both soybean Harosoy 63 and susceptible cultivar Harosoy. We also observed similar phenomena in our transgenic soybean roots. Thus we hypothesis that the gmSNF2 may sense the free sugar molecular from fungal wall released by soybean glucanases and thus somehow initiate a sugar mediated signal transduction pathway for pathogen resistance.

Construction of soybean genomic and a root cDNA library from *Phytophthora* resistant line L85-3044

For the cloning purpose, we have prepared both a genomic and cDNA library from a soybean line L85-3044 that possesses the *Phytophthora* resistance genes. This line is a near isogenic line to Williams. In addition, the cDNA library possesses gene products that may be induced by the presence of the fungus, since part of the mRNA population was derived from roots that were infected with soybean fungus *Phytophthora sojae.* Another advantage of these libraries is that they were prepared from size fractionated DNA populations. And the clones from

this cDNA library were excised into a pBK-CMV phagemid *in vivo* which can be express both in prokaryotic and eukaryotic cells

Increased efficiency of *Agrobacterium rhizogenes* mediated transformation of soybean seedlings and *Phytophthora* resistant screening in transgenic soybean roots

Agrobacterium mediated transformation efficiency varies with different soybean genotypes. Based on our present understanding of the gene silencing phenomena in transgenic plants and the fact that soybean genome has a high content of heterochromatin, we propose that the varying transformation efficiencies and levels of gene expression in soybean may be due to the presence of a rich heterochromatin environment and high level of methylation in the genome. To test this hypothesis, we treated soybean seedlings with the DNA methylation inhibitor 5-azacytidine as well as the synthetic plant hormone 2-4-D before transformation with *A. rhizogenes*. We observed a significant increase in the GUS reporter gene expression in soybean root hairs and about 4.5% of GUS gene insert were identified by PCR from hair roots without GUS expression, supporting our hypothesis. Statistical variance analysis of GUS expression data showed the two agents reduce the genotypic differences to non-significant levels. Furthermore, these two agents do not produce any detrimental effects on the soybean during development. This transient transformation system was to test *Phytophthora* resistance gene candidates. Because there is no highly efficient transformation system in soybean yet, and the conventional hypocotyl methods using inoculation methods with *Phytophthora* kills susceptible seedling eventually. This procedure is very useful. The coloration assay we developed does not kill susceptible individual plants. The transgenic roots can also be tested with the PCR and thin layer chromatography analysis. Using this system, we were able to test the

soybean gmSNF2 gene for complement and found that the susceptible soybean to have resistant reactions.

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