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Isolation and characterization of a Fusarium virguliforme toxin that induces foliar sudden death syndrome in soybean [Glycine max (L.) Merr.]

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Isolation and characterization of a *Fusarium virguliforme* toxin that induces foliar sudden death syndrome in soybean (*Glycine max* (L.) Merr.)

by

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A dissertation submitted to the graduate faculty

in partial fulfillment of the requirements for the degree of

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Major: Genetics

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2010

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ABSTRACT

*Fusarium virguliforme* (*Fv*) is the causal organism of the sudden death syndrome (SDS) in soybean (*Glycine max* [L.] Merr). The estimated average annual soybean yield loss from this disease is valued at about 300 million dollars. *F. virguliforme* is a soil borne fungus, which attacks soybean roots and causes root rot as well as chlorosis and necrosis in foliar tissues. The pathogen has never been isolated from the diseased foliar tissues. It was therefore hypothesized that a toxin(s) is involved in the development of the disease.

Cell-free *F. virguliforme* culture filtrate containing one or more toxins produces foliar SDS symptoms in cut soybean seedlings. Cell-free *F. virguliforme* culture filtrates were separated by conducting gel filtration and native PAGE and a 13.5 kDa, proteinaceous toxin FvTox1 was purified. Anti-FvTox1 monoclonal antibodies were raised against FvTox1 in mice and used in screening an expression cDNA library. A 516 bp long *FvTox1* cDNA was isolated from screening the cDNA library. *FvTox1* is a single gene containing two small introns (61 and 50 bp). *FvTox1* was expressed in an insect cell line with the aid of baculovirus. Expressed FvTox1 was able to produce chlorosis and necrosis in leaf discs of SDS-susceptible but not SDS-resistant soybean cultivars.

Two single chain variable fragment (scFv) antibody genes, *Anti-FvTox1-1* and *Anti-FvTox1-2*, were cloned from the hybridoma cell line expressing the anti-FvTox1 7E8 monoclonal antibody. *Escherichia coli* expressed Anti-FvTox1-1 and Anti-FvTox1-2 bound to FvTox1 on nylon membranes. *Anti-FvTox1* scFv antibody genes with KDEL sequence at their either 5’ or 3’ ends were cloned into a binary vector and four constructs were generated.
The *Agrobacterium rhizogenes* strains carrying these binary constructs were used to generate transgenic roots by infecting soybean cotyledons. Protein preparation from transformed hairy roots with all four constructs except the one carrying *Anti-FvTox1-1* with the KDEL sequence fused to its 5’-end were functional. The *Anti-FvTox1-1* gene with the KDEL sequence at its 3’end was used to generate stable transgenic soybean plants. The progenies of a transgenic plant carrying the *Anti-FvTox1-1* gene were investigated for foliar SDS development. Progenies carrying a copy of the transgene showed enhanced tolerance to the pathogen infection and FvTox1 as compared to the non-transgenic William 82 control plants that went through the same transformation procedures but carry no transgenes. These results suggested that FvTox1 is the pathogenicity factor that induces foliar SDS in soybean and expression of a plant anti-FvTox1 antibody could be a suitable strategy in enhancing foliar SDS resistance.

In order to understand the possible pathogenesis mechanism immunohistochemistry was conducted using the anti-FvTox1 7E8 monoclonal antibody. FvTox1 accumulates in the chloroplasts of the *F. virguliforme*-infected soybean plants suggesting that the site of FvTox1 action is in chloroplasts. The toxin may interfere the photosynthesis function to initiate foliar SDS symptoms as suggested by an earlier study.
CHAPTER 1 GENERAL INTRODUCTION

1. Introduction

Soybean (Glycine max (L.) Merr.) is one of the most important crops of the United States. The United States is the leading world soybean producer, 77 million metric tons; 35% of the total soybean produced globally (Willhite, 2004). In the year 2009, about 3.4 billion bushels were produced (USDA-NASS, 2010). Soybean also accounts for about 90% of total oil seed production in the United States (USDA-ERS, 2010). In 2005, the yield losses due to sudden death syndrome (SDS) equaled to 900 thousand metric tons (Wrather and Koenning, 2006). The disease is caused by a soil borne fungus Fusarium virguliforme (Fv), earlier known as Fusarium solani f. sp. glycine (Rupe, 1989; Aoki et al., 2003). The disease is characterized by root rot, chlorosis and necrosis of foliage, defoliation, shedding of leaves, flowers and pods (Hershman, 1990; Roy et al., 1997). The fungus attacks only the soybean roots and it has never been isolated from the above ground diseased tissues (Roy et al., 1989). The cell-free F. virguliforme culture filtrates are capable of producing SDS-like symptoms in cut soybean seedlings (Li et al., 1999). It is hypothesized that the pathogen releases a toxin(s) into the soybean roots that is translocated into the leaves, where it initiates foliar SDS. In this study the goal was to isolate and characterize the F. virguliforme toxin(s) that is involved in foliar SDS development.
2. Dissertation organization

This dissertation is presented in an alternative format and is composed of five chapters. Presented in Chapter 1 is a general introduction to SDS disease and proteinaceous toxins. In Chapter 2, isolation and characterization of a proteinaceous toxin, FvTox1 from the cell-free *F. virguliforme* culture filtrates is presented. In this chapter, data relating to 1) purification of the FvTox1 protein; 2) cloning of *FvTox1* by screening expression cDNA *F. virguliforme* library; 3) investigation of the *FvTox1* copy number; 4) cloning of *FvTox1* into baculovirus expression vector pFastBac1; and 5) development of the leaf disc assay for testing responses soybean towards expressed FvTox1 proteins are presented. I conducted all experiments except the baculovirus expression of FvTox1 presented in Chapter 2. In Chapter 3, data relating to the creation and expression of an Anti-FvTox1 single chain variable fragment antibody expressing gene is presented. I conducted all experiments except the electron microscopy and soybean transformation presented in this section. Chapter 4 describes the work on localization of FvTox1 in the infected soybean leaf tissues. Chapter 5, as a general conclusion chapter, summarizes all the results from Chapter 2 to Chapter 4.

3. Literature review

Toxins are more commonly produced by bacterial pathogens, but recently a number of fungal pathogens producing proteinaceous toxins have been discovered.

3.1 Sudden Death Syndrome

3.1.1 The disease

Sudden death syndrome (SDS) was first observed in Arkansas in the year 1971 (Hirrel, 1987). At present SDS is found in almost all the major soybean growing states in the United
States. At present about 15 states, including Iowa are affected by this disease (Roy et al., 1997; Malvick, 2006; Wrather and Koenning, 2006; Ziems et al., 2006). It was ranked fifth among all the major soybean diseases in the United States (Wrather and Koenning, 2006). Outside the United States, the disease is found in Argentina and Brazil (Rupe and Hartman, 1999).

The characteristic symptoms of SDS are root rot, crown necrosis, premature defoliation, and flower and pod abortion (Rupe, 1989). The symptoms of SDS can be divided into two components, leaf scorch and root rot. Symptoms are first visible as small chlorotic spots in the interveinal regions of the leaves. In the later stages of the disease chlorotic spots expand all over the leaf and turn into necrotic spots. In general the foliar symptoms start either at or immediately after flowering. In case of severe infection, the plants will defoliate and even the flowers and pods will fall off, leading to major yield losses (Rupe, 1989; Roy et al., 1997). In the root rot component, discoloration and rotting of the crown region is visible. If the infection reaches severe levels, the root system is heavily damaged. The nitrogen fixing nodules as well as the lateral roots fall off and sometimes the tap root is also heavily deteriorated (Hirrel, 1987; Hershman, 1990; Roy et al., 1997).

The disease causes a yield loss ranging from 5 to 80%, depending on the developmental stage of the plant at which the fungus attacks (Roy et al., 1997). It has been reported that the relationship between the SDS foliar symptoms and soybean yield reduction is linear (Luo et al., 2000). SDS symptoms are sometimes confused with Brown stem rot symptoms. However the two can be distinguished by looking at the browning of the stem pith which occurs in Brown stem rot and not in SDS (Hartman et al., 1999). SDS development is
also positively correlated with soybean cyst nematode infection (Roy et al., 1989; Roy et al., 1997).

SDS severity is affected greatly by the existing environmental conditions. Low temperatures, high moisture, high soil fertility and early planting, enhance the disease incidence (Hershman, 1990; Rupe et al., 1991; Gibson et al., 1994; Rupe et al., 1994; Roy et al., 1997).

3.1.2 The Causal Agent

SDS is caused by a soil borne fungus, *Fusarium solani* f. sp. *glycines* (Roy et al., 1989; Rupe, 1989). The causal agent has been recently renamed as *Fusarium virguliforme* (*Fv*) (Aoki et al., 2003). In this manuscript the causal agent of SDS will be referred as *F. virguliforme*. *F. virguliforme* is classified under fungi imperfecti, reproducing asexually (similar to ascomycetes) by producing conidiospores. The fungus produces macroconidia in abundance (Roy et al., 1997).

The fungus actually infects and resides on the underground parts of the soybean plant. Studies have shown that the fungus attacks the roots, but does not move upwards in the plant (Roy et al., 1989; Rupe, 1989; Roy et al., 1997). It has also been observed that the cell-free *F. virguliforme* culture filtrates can produce SDS like leaf scorch, when fed to three week old seedlings cut below the cotyledon (Li et al., 1999). Less diluted cell-free *F. virguliforme* culture filtrates cause severe symptoms in cut soybean seedlings (Hartman et al., 2004). The foliar symptoms can be produced even in the absence of the root system of the soybean plant (Roy et al., 1997; Li et al., 1999).
Presence of soybean cyst nematode (SCN) has also been positively associated with SDS occurrence (Rupe et al., 1991; Gibson et al., 1994; Rupe et al., 1994). The fungus *F. virguliforme* was first isolated from SCN in the year 1986 (Roy et al., 1997). The clamydospores of the fungus were found to be residing within SCN cysts. It is being thought that the spores of *F. virguliforme* overwinter in the SCN cysts and therefore causes a more intense SDS in the next growing season (Roy et al., 1997).

Apart from *F. virguliforme* which is responsible for causing SDS in North America, *Fusarium tucumaniae* has also been shown to cause SDS in soybean grown in South America (Aoki et al., 2003). While *F. tucumaniae* has two mating types and includes a sexual reproductive stage in its life cycle, all the samples of *F. virguliforme* are of the same mating type and hence it is concluded that the specie never reproduces sexually (Covert et al., 2007).

A high throughput multi locus assay was developed based on nucleotide polymorphism within the nuclear ribosomal intergenic spacer region rDNA and in two anonymous intergenic regions, locus 51 and 96. This assay was able to successfully differentiate between *F. tucumaniae* and *F. virguliforme* on allelic basis (O'Donnell et al., 2010).

It has been recently found that four phylogenetically distinct *Fusarium* species, *F. brasiliense* sp. nov., *F. cuneirostrum* sp. nov., *F. tucumaniae*, and *F. virguliforme*, can cause SDS disease in soybean (Aoki et al., 2005).

### 3.1.3 Breeding for Resistance

It has been more than a decade since efforts for determining the genetics of SDS resistance in soybean cultivars started. As the response of cultivars towards SDS changes drastically with the change in environmental conditions, results of greenhouse and field
experiments differ greatly. Under greenhouse screens the resistance against SDS was reported to be monogenic. A single dominant gene, $R_{fs}$ from cultivar Ripley has been found to impart resistance against SDS under greenhouse conditions (Stephens et al., 1993). However, the effect of $R_{fs}$ gene was partial under field conditions (Gibson et al., 1994). Under the field conditions genetics of SDS resistance has been reported to be partial and polygenic in nature (Mathews et al., 1991; Gibson et al., 1994; Hnetkovsky et al., 1996; Njiti et al., 1996). Fourteen QTL conferring SDS resistance have been identified. One QTL each has been detected in linkage group (LG) A2 (Hashmi, 2004), LG F (Kassem et al., 2006), LG I (Iqbal et al., 2001), LG J (Kassem et al., 2006) and LG L (Hashmi, 2004; Njiti and Lightfoot, 2006). Two QTL each are located on the LG C2 (Hnetkovsky et al., 1996; Njiti et al., 1998; Iqbal et al., 2001; Njiti et al., 2002), LG D2 (Lightfoot et al., 2001; Farias Neto et al., 2007) and LG N (Chang et al., 1996; Njiti et al., 1998; Njiti et al., 2002; Hashmi, 2004). LG G has three QTL (Chang et al., 1996; Njiti et al., 1998; Meksem et al., 1999; Prabhu et al., 1999; Iqbal et al., 2001; Njiti et al., 2002). Separate QTL have been recognized for foliar and root SDS resistance (Kazi et al., 2008). Due to the fact that multiple loci contribute towards SDS resistance, effective selection experiments must be conducted at multiple environmental locations (Njiti et al., 2001). There has been a general lack of efforts towards incorporating SDS resistance genes into the susceptible soybean cultivars, because vertical SDS resistance genes have not been identified.

3.2 Toxins of Fungal Origin

Small molecular weight proteins/toxins, secreted by plant pathogenic fungi have been shown to cause diseases (Rep, 2005). Some toxins are host selective toxins (HSTs) because of their
abilities to cause disease only in certain host plants. So far about 20 HSTs have been discovered, majority of them are either low molecular weight metabolites or proteins. All known proteinaceous HST’s are encoded by single genes. The most common defense response of the host plants against a pathogen occurs via production of reactive oxygen species, cell wall alterations, hypersensitive response and production of pathogen related proteins (Wolpert et al., 1994). In diseases produced by HST’s, host cell death is likely to contribute towards susceptibility (Wolpert et al., 2002).

3.2.1 Non-Proteinaceous Fungal Toxins

_Fusarium_ species are one of the most virulent toxin-producing fungi (Creppy, 2002). The toxins produced by _Fusarium_ effect either animals (Zearalenone) or plants (Fusaric acid and Phytolycoperin), and in some cases both plants and animals (Enniatins, Beauvericins, Moniliformin and Fumonisins produced by _F. verticillioides_) (Desjardins and Hohn, 1997). Certain _Fusarium_ species produce trichothecenes, which are very strong phytotoxins (Desjardins and Hohn, 1997). It has been found that a number of _Fusarium_ species are capable of producing toxins, some of which consist of one or more peptides.

The toxins produced by _Cochliobolus_ spp. (HC toxins, T-toxins and Victorin) in particular have been found to be very important in pathogenesis. Victorin, a 8 kDa, cyclic pentapeptide, is another host specific toxin produced by fungus _C. victoriae_, which causes Victoria blight of oats (Meehan and Murphy, 1946). In the fungal culture filtrates, the toxin is found in 5 naturally occurring forms, with form C contributing 85-90% total activity (Macko et al., 1985; Wolpert et al., 1985). It has been found that victorin sensitivity in oats is controlled by a dominant gene _Vb_, the homozygous recessives being insensitive to victorin
and hence resistant to the pathogen. The same gene also imparts resistance against crown rust of oats caused by *Puccinia coronata*. The homozygous recessive genotypes are susceptible to crown rust (Scheffer and Livingston, 1984).

In *in vivo* experiments, victorin binds to the 100 kDa P protein component of glycine decarboxylase complex of the susceptible oat genotypes (Wolpert et al., 1994). Victorin also binds to a 15 kDa mitochondrial protein, which constitutes the H protein component of glycine decarboxylase complex. This binding to the 15 kDa protein occurs in both susceptible and resistant genotypes of oats (Navarre and Wolpert, 1995). In conclusion victorin leads to inhibition of glycine decarboxylase complex, which is a part of photorespiratory cycle.

An apoptosis like response was observed and confirmed via observation of DNA laddering, cell shrinkage, altered mitochondrial function and substrate specific proteolytic events in the victorin treated leaves (Navarre and Wolpert, 1999). The sensitivity of *Arabidopsis thaliana* to victorin is controlled by a single dominant locus *LOV1*, a CC-NB-LRR, localized on the north arm of Chromosome 1, with the dominant gene being associated with disease susceptibility (Lorang et al., 2004).

### 3.2.2 Proteinaceous Fungal Toxins

Most proteinaceous toxins are small molecular proteins and are classified as elicitors of host cell death induction. A majority has been known to be nonspecific and only a few have been classified as the host-specific. For example, the necrosis inducing protein (NIP1), from a barley pathogen *Rhynchosporium secalis* is encoded by the avirulence gene, *AvrRrs1* recognized by the barley resistance protein *Rrs1* (Roche et al., 1995). Two 5.6 kDa and 5.8
kDa peptide elicitors have been isolated from cowpea rust fungus *Uromyces vignae*. They are similar in size, but do not have any similarities to the known peptides. Both peptides are capable of eliciting the hypersensitive response in cowpea (D'Silva and Heath, 1997). An 18.5 kDa protein (VD18.5) isolated from the *Verticillium dahliae* culture filtrates has been shown to cause disease symptoms, typical of *Verticillium* wilt disease in cotton. The host specificity of this phytotoxin has not yet been established (Palmer et al., 2005).

Four proteinaceous toxins were isolated from the *Fusarium spp*. Two of these toxins (56 kDa and 61 kDa), isolated from *F. oxysporum* f. sp. *lycopersici* race 1, cause cell death in protoplasts prepared from susceptible tomato cultivars (Sutherland and Pegg, 1995). The third one (24 kDa), from *F. oxysporum* f. sp. *erythroxli*, was named nep1 as it causes necrosis and ethylene production when it is applied to the leaves of a wide variety of dicotyledons (Bailey, 1995; Bailey et al., 2000). A 17 kDa protein was purified from *F. virguliforme*, but it has not has been experimentally shown to cause SDS (Jin et al., 1996).

Only a few proteinaceous toxins have been categorized as HST. Cerato-ulmin (CU) is a hydrophobic protein produced by *Ophiostoma ulmi*, an ascomycete fungus that causes Dutch elm disease (Temple et al., 1997). The purified protein causes symptoms in elm seedlings similar to those caused by the fungus. The levels of the CU protein in the culture filtrates of *O. ulmi* were shown to be associated with to the virulence (Takai, 1974; Scala et al., 1997). The *cu* gene is interrupted by two introns and encodes for a 100 amino acid long prepro-CU. The prepro-CU is further processed into a 75 amino acid long mature protein (Bowden et al., 1994).

Two 35 kDa HSTs, AB and AP toxins from *Alternaria brassicola* and *Alternaria panax*, respectively, were isolated from germinating spore fluids (Otani et al., 1998;
Quayyum et al., 2003). Both toxins cause necrosis. A 12.4 kDa phytotox protein, cerato-platanin (CP) causes necrosis and accumulation of fluorescent compounds in leaves of tobacco and plane trees (*Platanus acerifolia*). The CP is produced by an ascomycete fungus, *Ceratocystis fimbriata*, the causal agent of canker stain disease (Pazzagli et al., 1999; Pazzagli et al., 2006).

Many proteinaceous HSTs are produced by the fungus *Pyrenopora tritici-repentis* (*Ptr*), responsible for causing tan spot disease in wheat. The necrosis causing toxin, *Ptr ToxA* was independently isolated from *Ptr* culture filtrates by four different groups (Ballance et al., 1989; Tomas et al., 1990; Tuori et al., 1995; Zhang et al., 1997). *Ptr ToxA* gene encoding the *Ptr ToxA* has been isolated (Ciuffetti et al., 1997).

Genomic organization of the 1,319 bp *Ptr ToxA* cDNA clone revealed that the locus consists of an endogenous promoter, a 22 amino acid long N-terminal signal peptide, 38-39 amino acid long N terminal domain and a 117-118 amino acid long C-terminal domain. There is one intron each in the N and C terminal domains. The active secreted form of the toxin consists of the C terminal domain only (Ciuffetti et al., 1997). N terminal domain, presumably involved in protein folding, was involved in increasing the specific activity of *Ptr ToxA*. An RGD (Arginine, Glycine, and Asparagine) cell attachment motif, similar to the mammalian protein Vitronectin, was detected in *Ptr ToxA*. Mutations in this motif lead to reduction of the toxin activity (Manning et al., 2004; Manning et al., 2008).

*Ptr ToxB* was isolated from *Ptr* and shown to induce chlorosis in susceptible wheat cultivars. *Ptr ToxB* is a 6.61 kDa protein, heat stable and its minimum active concentration is 14 nM (Strelkov et al., 1999). *Ptr ToxB* is encoded by a multi-copy gene, *ToxB* (Martinez et al., 2001; Martinez et al., 2004).
Three more toxins have been isolated from *Ptr* culture filtrates, but have not been characterized as proteins. *Ptr* ToxC has been isolated from culture filtrates of *Ptr* and shown to be a low molecular weight molecule. The chemical nature of the molecule has not yet been established. *Ptr* ToxC causes chlorosis in wheat cultivars (Effertz et al., 2002). *Ptr* ToxD was isolated independently by two groups. One was isolated from race ARD1 of Argentina (Ali et al., 2002), the second one was isolated in race SO3 (Manning et al., 2002). It is not known if the two *Ptr* ToxDs are the same molecule.

Five proteinaceous toxins have been identified from the wheat pathogen *Stagonospora nodorum* that causes leaf and glume blotch disease in wheat (Lui et al., 2004; Friesen et al., 2006; Friesen et al., 2007; Friesen et al., 2008; Abeysekara et al., 2009). Among these toxins, SnToxA showed high similarity to *Ptr* ToxA. *Ptr* ToxA, with identity greater than 99% to SnToxA, is believed to have emerged from interspecific gene transfer (Friesen et al., 2006). Transfer of this single toxin gene, *Ptr* ToxA, form *Stagonospora nodorum* to *P. tritici-repentis*, is believed to lead the emergence of tan spot disease in wheat (Friesen et al., 2006). This study signifies the role of toxins in plant disease development.

### 3.3 Plant Antibodies

An antibody molecule is a Y shaped structure consisting of two heavy (H) and two light (L) chains (Davies and Chacko, 1993). The arms of the Y shaped molecule constitute the Fab (antigen binding fragment) region and the stem constitutes the Fc (constant fragment) region. Both the H and the L chains consist of variable region (VH and VL respectively) and the constant regions (CH and CL respectively). The N terminal ends of the VH and the VL region govern the antigen binding specificity (Tonegawa, 1983; Davies and Chacko, 1993).
The whole antibody molecule is complex and held together with various disulfide bonds and other intra-molecular interactions. Therefore cloning and expression of the entire antibody molecule in foreign organisms can pose various problems related to proper folding of the antibody molecule in the target organisms. Whole recombinant molecules are seldom found to be active in the bacterial system and only a very small percentage of molecules are active when expressed in viruses (Wörn and Plückthun, 2001). Despite of many difficulties in expression of the entire antibody molecules, there have been studies where whole antibody molecules have been successfully expressed in target organism. The whole antibody molecule against phosphate ester has been successfully expressed in tobacco. The antibody constitutes 1.3% of the total leaf extract proteins (Hiatt et al., 1989). Full length monoclonal antibodies against herpes virus produced in soybean have been shown to function similarly to the original antibody molecule (Zeitlin et al., 1998). But studies demonstrating successful expression of the entire antibody molecules are limiting.

In view of these problems in expression of the entire antibody molecules, priority has been given to cloning of the variable domains as single chain antibodies [single chain variable fragment (scFv)]. ScFv antibodies consist of VH and VL regions, joined by a 15 amino acid long flexible polylinker consisting of glycine and serine residues. Another major advantage of expression of scFv antibodies instead of the whole molecule is the ease of passage of scFv molecules through membranes. scFv molecules being smaller in size can be easily purified as compared to the whole antibody molecules (Bird et al., 1988).

A major discovery that has made the synthesis of scFv antibodies possible is the hybridoma technology, where monoclonal antibodies against almost any antigen can be produced (Kohler and Milstein, 1975). Antibody coding genes can be cloned from the
mRNA derived from hybridoma lines producing the antibody. The primers can be designed based on the conserved end sequences of the mature VL and VH chains. Certain restriction sites are rarely present in the antibody coding genes; therefore these rare restriction sites can be used in cloning the antibody genes into expression vectors (Orlandi et al., 1989; Chaudhary et al., 1990).

In numerous studies scFv molecules have been successfully expressed. It has been shown that it is advantageous if the VH and VL chains are encoded by a single DNA fragment. This increased the yields in *E. coli* to 9% (Huston et al., 1988). Chimeric antibody with variable regions form mouse antibody and constant regions from the human antibodies have been assembled and expressed in *E. coli* (Better et al., 1988). An approach to create immunotoxins was developed using the concept of scFv antibodies. A scFv cloned from the antibody against cancer cells was fused with an exotoxin gene to kill specifically the ovarian cancer cells. This antibody was shown to be functional in *E. coli* (Chaudhary et al., 1990).

A scFv against Zearalenone (*Fusarium* toxin) has been expressed in transgenic *Arabidopsis* plants. The expressed scFv was guided via a signal peptide and found distributed in all the electron dense organs of the plant cell (Yuan et al., 2000). A scFv antibody has been developed against the coat protein of *Citrus tristeza virus* (CTV) and this scFv has been expressed in *E. coli* (Galeffi et al., 2002).

In a recent study, a scFv against human epidermal growth factor was cloned from hybridoma cell lines and stably expressed in *E. coli* and in tobacco and *Nicotiana benthamiana* (Galeffi et al., 2002). Addition of an amino acid sequence for retention in endoplasmic reticulum, KDEL to the c-terminus of the expressed proteins helps to localize
and stabilize them in the plant cytosol. However this does not hold true for all the scFv antibodies (Schouten et al., 1997).

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CHAPTER 2 FOLIAR SUDDEN DEATH SYNDROME IN SOYBEAN IS DEVELOPED BY A CULTIVAR-SPECIFIC PHYTOTOXIN PRODUCED BY *Fusarium virguliforme*

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Abstract

Sudden death syndrome (SDS), caused by *Fusarium virguliforme* (*Fv*) is a serious fungal disease in soybean. The pathogen has never been found in diseased foliar tissues. A phytotoxin(s) has been implicated in causing foliar SDS. Cell-free *Fv* culture filtrates contain a proteinaceous phytotoxin that causes foliar SDS in cut soybean seedlings. To purify the phytotoxin, cell-free *Fv* culture filtrates were subjected initially to gel filtration and then to native polyacrylamide gel electrophoresis. A ~13.5 kDa, low-molecular mass protein (*FvTox1*) was purified from *Fv* culture filtrates that produced foliar SDS in cut soybean seedlings. Anti-*FvTox1* monoclonal antibodies raised against the purified *FvTox1* was used in isolating *FvTox1* from an expression cDNA library. Recombinant *FvTox1* protein expressed in an insect cell line resulted in chlorosis and necrosis that are typical foliar SDS symptoms. SDS-susceptible, but not the SDS-resistant, soybean lines were sensitive to the expressed toxin, suggesting that *FvTox1* is cultivar-specific and involved in foliar SDS development.
Introduction

Sudden death syndrome (SDS) is a serious emerging soybean disease in the United States (Wrather and Koenning, 2006). It is caused by the soil-borne fungal pathogen, *Fusarium virguliforme*, previously known as *F. solani* f. sp. *glycines* (Roy et al., 1989; Rupe, 1989; Roy et al., 1997). In the United States, the disease was reported first in Arkansas in 1971 (Hirrel, 1987). Subsequently, the pathogen has gradually spread to the north, and recently it has been identified in Minnesota and Nebraska (Malvick, 2006; Ziems et al., 2006). To date, at least 15 states are affected by this disease (Roy et al., 1997; Malvick, 2006; Wrather and Koenning, 2006; Ziems et al., 2006). In leaves, the disease progresses as interveinal chlorosis and necrosis. In more advanced cases, shedding of leaves, flowers and pods leads to severe yield suppression (Hirrel, 1987; Hershman, 1990; Roy et al., 1997).

Resistance of soybean cultivars against *F. virguliforme* is partial (Mathews et al., 1991; Gibson et al., 1994; Hnetkovsky et al., 1996; Njiti et al., 1996). A single dominant gene *Rfs* from cultivar Ripley was found to impart resistance against SDS under greenhouse conditions (Stephens et al., 1993). However the effect of *Rfs* gene was partial under field conditions (Gibson et al., 1994). Several QTL control SDS resistance (Iqbal et al., 2002; Njiti et al., 2002).

In South America, *Fusarium tucumaniae* causes SDS in soybean (Aoki et al., 2003). Both *F. virguliforme* and *F. tucumaniae* are closely related species with little variation at the nucleic acid level, but the two species are morphologically and phylogenetically distinct (O’Donnell, 2000; Aoki et al., 2003). Both species attack soybean roots and cause deterioration of the root system. Neither pathogen species has ever been found in above-ground diseased tissues (Roy et al., 1989; Rupe, 1989). Based on loci 51 and 96, *F.*
virguliforme was differentiated from *F. tucumaniae* (O'Donnell et al., 2010). *F. tucumaniae* has two mating types; and therefore, it has a sexual reproductive stage. *F. virguliforme* has been reported to have only a single mating type; and hence, it unlikely has a sexual reproductive stage (Covert et al., 2007). Although *F. virguliforme* is the main causal organism of SDS, it was recently reported that three additional, phylogenetically distinct, *Fusarium* spp. can cause this disease (Aoki et al., 2005).

*Fusarium* species are one of the most virulent toxin-producing fungi (Creppy, 2002). Host-selective toxins (HSTs) have been shown to cause diseases in susceptible genotypes. To date, 20 fungal pathogens have been shown to produce HSTs. The majority of the HSTs are either low-molecular-weight metabolites or proteins (Wolpert et al., 2002). Many of the fungal toxins are proteinaceous; and only a few are non-peptidic (Goudet et al., 1999).

Many proteinaceous fungal toxins have been studied in detail. Some of the known proteinaceous fungal toxins are: VD18.5 produced by *Verticillium dahliae* (Palmer et al., 2005); AB toxin of *Alternaria brassicola* (Otani et al., 1998); AP toxin of *Alternaria panax* (Quayyum et al., 2003); cerato-platanin from *Ceratocystis fimbriata* (Pazzagli et al., 1999); toxins from *F. oxysporum* f. sp. *lycoperscii* race 1 (Sutherland and Pegg, 1995); nep1 toxin from *F. oxysporum* f. sp. *erythroxli* (Bailey, 1995); Ptr ToxA and Ptr ToxB produced by *Pyrenophora tritici-repentis* (Ptr) (Ballance et al., 1989; Tomas et al., 1990; Tuori et al., 1995; Zhang et al., 1997; Martinez et al., 2001); and the five proteinaceous toxins identified from *Stagonospora nodorum* (Liu et al., 2004; Friesen et al., 2006, 2007, 2008; Abeysekara et al., 2009).

The facultative pathogen, *F. virguliforme* (*Fv*) can be grown in culture media. It excretes several proteins into liquid culture media. Cell-free *Fv* culture filtrates prepared
from liquid culture media after growing the pathogen for 12 days produces foliar SDS symptoms in three-week-old cut soybean seedlings (Li et al., 1999). It is considered that phytotoxin(s) released by *F. virguliforme* (*Fv*) to the soybean roots is involved in foliar SDS development, because the pathogen has never been detected in diseased foliar tissues (Roy et al., 1989; Rupe, 1989; Li et al., 1999). A 17 kDa protein was partially purified from *F. virguliforme* culture filtrates that causes necrosis in soybean tissues (Jin et al., 1996). However, the gene encoding the toxin was never isolated and it was not experimentally established if the purified protein was the toxin that induces foliar SDS in soybean. Here we report identification of the single gene, *FvTox1* that encodes a 13.5 kDa acidic protein, FvTox1. The native FvTox1 of the cell-free *Fv* culture filtrates and FvTox1 expressed in an insect cell line produced chlorosis and necrosis in SDS-susceptible soybean cultivars. The SDS susceptible, but not the resistant, soybean lines were sensitive to FvTox1. These results suggested that FvTox1 is a major pathogenicity factor for foliar SDS development in soybean.

**Results**

**Purification of Fv Toxin by Gel Filtration**

We applied a previously reported bioassay for purifying a candidate phytotoxin(s) released by *F. virguliforme* into culture filtrates (Li et al., 1999; Ji et al., 2006). Lyophilized cell-free *Fv* culture filtrate was separated in a Sephacryl S300HR column and 1-mL fractions were evaluated for possible phytotoxic activity. Fractions 33 to 57 produced SDS symptoms in cut soybean seedlings. Fractions 38-46 caused the most severe foliar SDS symptoms. The phytotoxic activities of the fractions 33-57 were associated with the protein peak of the
column fractions (Figure 1A) and that of the fractions 38-46 were with a low-molecular-weight protein (Figure 1B). These results suggest that the phytotoxin(s) produced by Fv could be proteinaceous.

**Proteinase K Treatment Abolished the Phytotoxic Activity of Cell-Free Fv Culture Filtrates**

Proteinase K is an endolytic protease that cleaves peptide bonds at the carboxylic sides of aliphatic, aromatic or hydrophobic amino acids. Following Proteinase K treatment, lyophilized cell-free Fv culture filtrate was separated in a Sephacryl S300HR column and 1-mL fractions were collected. Proteinase K treatment degraded the proteins of the Fv culture filtrate. Only a single protein band (27 kDa), presumably Proteinase K, was observed in the Proteinase K-treated sample (Supplemental Figure 1). Column fractions of the Proteinase K-treated cell-free Fv culture filtrate sample failed to produce any foliar SDS symptoms. These results suggested that the Fv toxin(s) is proteinaceous.

**A Low Molecular Weight Protein Caused Foliar SDS**

Column fractions showing phytotoxic activities (Figure 1A) were pooled, lyophilized and then separated on native-polyacrylamide gels. The gels were fractionated into six horizontal fractions, I-VI; and proteins of individual fractions were eluted for determining their possible phytotoxic activities (Figure 2A). Eluted proteins from fractions I to V did not produce any symptoms (Figure 2B); whereas fraction VI was able to produce foliar SDS symptoms in stem-cutting assay (Figure 2C). The low-molecular-weight protein of this
fraction is most likely the protein molecule of column fractions 38-46 shown with an arrow in Figure 1B. The purified protein from faction VI was resolved in both native and denaturing gels. In the native gel, a single <10 kDa protein was observed (Figure 3A); whereas, in the denaturing gel the estimated size was ~13.5 kDa (Figure 3B).

**Generation of Monoclonal Anti-FvTox1 Antibodies that Suppressed the Phytotoxic Activity of Fv Culture Filtrates**

The purified FvTox1 (Figure 3) was used to raise polyclonal antibodies in mice. The sera from four mice were tested for cross-reactivity to FvTox1 in western blot analyses. Polyclonal antibodies of one mouse showed strong cross-reactivity to FvTox1 (Figure 4A). The splenocytes from this mouse were fused to the mice myeloma cell line to create hybridoma clones. Through ELISA and western blot analyses, extracts from hybridoma cell lines were tested for their possible cross-reactivity to FvTox1 (Figure 4B). Of the four hybridoma clones selected, 7E8 and 13F5 showed stronger cross-reactivity to FvTox1 than that by 2F3 and 15C2 (Figure 4B). The monoclonal antibodies were investigated for their specificities to phytotoxin(s) of the Fv culture filtrate in immuno-suppression experiments. In the presence of increasing amounts of the antibodies, the foliar SDS symptom development was reduced. Maximum reduction in symptom development was seen when 7E8 antibody was used in immuno-suppression experiments (Supplemental Figure 2). These results suggested that monoclonal antibodies were specific to a phytotoxin(s) of the cell-free Fv culture filtrates that induces foliar SDS.
**Isolation of a cDNA Encoding FvTox1**

As a first step towards isolating the *FvTox1* gene, the N-terminal end of the purified FvTox1 was sequenced. The sequence was then utilized to PCR amplify the *FvTox1* gene. We failed to PCR amplify any products. We then applied immunoscreening procedure to clone the gene. An expression cDNA library in λTriplEx2 vector was screened with a mixture of all four ant-FvTox1 monoclonal antibodies (Figure 4B). Three putative cDNA clones were isolated from screening approximately 500,000 plaque forming units. These clones were sequenced and the sequences were investigated for the presence of the N-terminal sequence obtained earlier. One of the clones was found to contain the N-terminal sequence of FvTox1. This clone was induced by isopropyl-β-D-thiogalactoside (IPTG). Protein expressed by this clone was recognized by all four monoclonal anti-FvTox1 antibodies (data not shown).

**FvTox1 is a Novel Protein**

Sequence of the putative *FvTox1* cDNA was analyzed using the NCBI Blast program, BLASTX (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The FvTox1 showed high similarities to four hypothetical proteins of *Nectria haematococca* (EEU40946), *Fusarium oxysporium* (FOXG_06271.2), *Fusarium verticillioides* (FVEG_04124.2) and *Gibberella zeae* (XM_389746) (Supplemental Figure 3). The deduced FvTox1 (pro-FvTox1) protein has an isoelectric point of 4.47 and a molecular mass of ~20 kDa (Expasy Findmod tool; http://ca.expasy.org/tools/findmod/). Through comparison of the N-terminal sequence of the purified FvTox1 (mature-FvTox1) with the deduced pro-FvTox1 sequence it was evident that N-terminal 32 aa are cleaved off from the pro-FvTox1 prior to secretion of the mature-
FvTox1 into culture media The calculated molecular mass of the mature-FvTox1 was 15.5 kDa, similar to the estimated MW ~13.5 kDa based on mobility of the purified protein in a denaturing gel (Figure 3).

**FvTox1 is a Single Gene**

A genomic library was constructed from Fv genomic DNA and seven clones were identified. All seven clones showed identical restriction patterns suggesting that they were representative clones of the same FvTox1 gene (data not shown). The 3-kb fragment obtained from a genomic FvTox1 clone was sequenced and analyzed using FGENESH 2.6 Prediction program (Salamov and Solovyev, 2000). The TATA box was found to be located 135 bp upstream of the start codon. The gene contains two small introns of 61 and 50 base pairs (Figure 5A). Both introns contain consensus eukaryotic splice sites (5’-GT and 3’-AG) (Gurr et al., 1987). The predicted exon/intron boundaries were confirmed by comparing the gene sequence with the cDNA sequence.

The copy number of FvTox1 was investigated by conducting low stringency Southern blot analysis and by analyzing the F. virguliforme genome sequence. Southern hybridization experiments suggested that FvTox1 is a single copy gene (Figure 5B). The genome of Fv was sequenced using a Solexa genome analyzer II. Twenty genome equivalents sequence was investigated for possible FvTox1 homologs (http://blast.ncbi.nlm.nih.gov/Blast.cgi). We did not identify any genes similar to FvTox1.
Expressed FvTox1 Proteins Caused Chlorosis and Necrosis in Soybean Leaf Discs

In order to establish function of the candidate *FvTox1* gene, it was expressed in an insect cell line, Sf21. Both *pro-FvTox1* (encoding the entire ORF; protoxin) and *mature-FvTox1* (encoding the secreted peptide; mature toxin) were cloned into the expression vector pFastBac1 (Invitrogen, Carlsbad, CA). The recombinant FvTox1 proteins isolated from the baculovirus infected insect cell line (Figure 7) were infiltrated into soybean leaf discs and incubated under continuous light for four days. Initially two soybean cultivars, Essex and Forrest, susceptible and partially resistant to *F. virguliforme*, respectively, were investigated for their responses to the expressed FvTox1 proteins. The typical foliar chlorotic SDS symptom was observed when the FvTox1 proteins were infiltrated into leaf discs of the SDS-susceptible cultivar, Essex (Figure 8A). Leaf discs infiltrated with cloned mature- and pro-FvTox1 produced browning in addition to chlorosis suggesting possible generation of necrotic cells (Figure 8A). Severe damages in Essex leaf discs as compared to that in Forrest leaf discs were observed (Supplemental Figure 4).

We investigated if chlorotic symptoms induced by the FvTox1 proteins were associated with susceptibility of soybean cultivars to *F. virguliforme*. Leaf discs of four SDS-resistant and four SDS-susceptible lines including, Essex and Forrest, were vacuum infiltrated with recombinant FvTox1 proteins. Consistently, all four SDS-resistant cultivars showed tolerance to expressed FvTox1 proteins. On the contrary, chlorosis was pronounced in leaf discs of the SDS-susceptible cultivars (Supplemental Figure 5). Chlorophyll contents were significantly reduced in the SDS-susceptible but not in the SDS-resistant soybean cultivars infiltrated with FvTox1 proteins (Figure 8B). Induction of chlorotic SDS symptoms in leaf discs of SDS-
susceptible but not in SDS-resistant lines suggested that FvTox1 identified in this investigation is a key pathogenicity factor for SDS development in susceptible soybean lines.

Relative Toxicities of Mature-FvTox1 and Pro-FvTox1 Forms

Although the expression of mature-FvTox1 was not detectable in PAGE gel stained with Coomassie blue (Figure 7A), protein preparations from the insect cell line carrying the mature-FvTox1 gene were routinely producing chlorosis in SDS-susceptible soybean cultivars. To determine the relative phytotoxic activities of pro-FvTox1 and mature-FvTox1 forms, we compared the effect of similar amounts of pro-FvTox1, mature-FvTox1 and FvTox1 from cell-free Fv culture filtrates in soybean leaf-discs assays (Figure 9). Significantly reduced phytotoxic activities of pro-FvTox1, as compared to that of mature-FvTox1 and FvTox1 from Fv culture filtrates, were observed consistently among four SDS-susceptible lines (Figure 9B).

Different Cleavage Sites of FvTox1 in Fungus and Insect Cell Lines

We investigated if N-terminal signal peptide of FvTox1 is cleaved in the insect cell line, Sf21. A His-tag was fused to the N-terminus of pro-FvTox1 and expressed in Sf21. Western blot analyses using monoclonal anti-FvTox1 antibody (7E8) and anti-His antibody showed that N-terminus of pro-FvTox1 was cleaved in the insect cell line (Figure 10A). To determine the cleavage site of the signal peptide, we conducted N-terminal sequencing. The first 19 amino acids of the pro-FvTox1 were found to be excised in insect cells (Figure 10B). The processed FvTox1 was secreted to the liquid-cell-culture media (Figure 10C). The
cleavage site of expressed Fvtox1 in the insect cell line matched the predicted site by a number of bioinformatics programs (SignalP 3.0 Server; (Dyrløv Bendtsen et al., 2004)).

Discussion

The FvTox1 Phytotoxin Causes Foliar SDS in Soybean

*Fusarium virguliforme*, causal organism of soybean sudden death syndrome, has never been isolated from diseased foliar tissues. It has been speculated that one or more phytotoxins released by the pathogen into the infected roots cause foliar SDS symptoms. The facultative pathogen *F. virguliforme* also releases the phytotoxin(s) to the culture media. In this investigation we could isolate only a single peptide, FvTox1 from the Fv culture filtrate that meets the three criteria for a phytotoxin (Scheffer, 1983). When expressed in an insect cell line, FvTox1 caused loss of chlorophylls as well as necrosis in soybean leaf discs. The loss of chlorophyll is a typical symptom of foliar SDS (Ji et al., 2006). Cell-free Fv culture filtrates containing this phytotoxin cause foliar SDS symptoms only in presence of light (Ji et al., 2006). Similarly, FvTox1 isolated in this investigation can cause necrosis only in the presence of light (Supplemental Figure 6). SDS-susceptible soybean cultivars, not the SDS-resistant cultivars, were highly sensitive to FvTox1 (Figure 8). These functional data suggested that FvTox1 is a major pathogenicity factor of *F. virguliforme* involved in foliar SDS development in soybean.

Role of FvTox1 in Foliar SDS Symptom Development

Induction of host cell death machinery is one of the mechanisms used by fungal pathogens to overcome the plant defense systems. Phytotoxins are shown to trigger
programmed cell death (PCD) leading to large scale chlorosis and necrosis (Rep, 2005). For example, victorin, a cyclized pentapeptide toxin, produced by *Cochliobolus victoriae* induces a form of PCD, similar to apoptosis, with DNA breakdown, shrinking cells and activation of proteases in oats (Wolpert et al., 2002). Sensitivity of Arabidopsis to victorin is mediated by a disease resistance (R)-like protein containing nucleotide binding and leucine rich region (NB-LRR) domains (Lorang et al., 2007). Similarity of molecular phenotypes induced by host-selective fungal phytotoxins in at least some plant-pathogen interactions with those by avirulence factors and identification of an R-like victorin-interacting protein suggests similarities between molecular mechanisms used by avirulence factors and toxins in initiating disease symptoms (Wolpert et al., 2002; Lorang et al., 2007).

Requirement of light in inducing foliar SDS symptoms by FvTox1 (Supplemental Figure 6) suggests that most likely the toxin interacts with one or more soybean proteins to initiate foliar SDS. Ptr ToxA, which shares functional similarity to FvTox1, has been localized to the chloroplast and interacts with a chloroplast protein (Manning and Ciuffetti, 2005; Manning et al., 2007). It induces reactive oxygen species (ROS) in a light-dependent manner and degrades chloroplast protein, Rubisco and brings changes to photosystem I and photosystem II (Manning et al., 2009). *Fv* culture filtrates containing FvTox1 was also shown to degrade Rubisco large subunit and produce ROS in a light dependent manner (Ji et al., 2006). The striking similarities between molecular disease phenotypes caused by the two similar proteinaceous phytotoxins, Ptr ToxA and FvTox1, suggest a common mechanism used by the two toxins in initiating programmed cell death (PCD) and diseased phenotypes.
Creating Transgenic Soybean Lines with Enhanced Foliar SDS Resistance

Most host selective toxins produced by fungi are low molecular weights and mobile. Therefore, symptoms can appear at a distance, far from the site of infection (Walton, 1996). FvTox1, released by the fungus to the roots, is likely transported through the vascular system to the leaves, where it causes foliar SDS. We hypothesize that once the toxin reaches the leaves, it interacts with one or more host proteins, possibly located in the chloroplast, to initiate degradation of Rubisco large subunits and ultimately PCD (Ji et al., 2006). Identification of the FvTox1-interacting soybean protein(s) will provide clues for a better understanding of the mechanism used by FvTox1 to cause SDS.

Sensitivity of SDS-susceptible but not SDS-resistant soybean cultivars to FvTox1 (Figure 8) suggested that FvTox1 functions in a cultivar-specific manner. Whether there is any variation among F. virguliforme isolates for FvTox1 production is yet to be investigated. Considering FvTox1 as a major pathogenicity or virulence factor, one could express anti-FvTox1 antibody in transgenic soybean plants to neutralize the destructive power of this phytotoxin in order to alleviate foliar SDS development. Foliar SDS is the major reason for yield reduction in F. virguliforme infected SDS-susceptible soybeans lines. It is conceivable that such lines with expressed anti-FvTox1 antibody will have reduced foliar SDS development as compared to nontransgenic control plants; as a result, the transgenic lines will continue to supply photosynthates to the roots and assist the plant to better fight the pathogen in infected roots tissues.
Materials and methods

Cell-Free *F. virguliforme* Culture Filtrates

Isolates of *F. virguliforme* (Fv) were grown on solid Bilay medium [(0.1% KH$_2$PO$_4$ (w/v), 0.1% KNO$_3$ (w/v), 0.05% MgSO$_4$ (w/v), 0.05% KCl (w/v), 0.02% starch (w/v), 0.02% glucose (w/v), and 0.02% sucrose (w/v)]. 40 plugs were transferred from the plate to 100 mL liquid modified Septoria medium (MSM) and incubated at room temperature for 12 days in the dark without shaking. The liquid was passed through two layers of Whatman No.1 filter paper and pH was adjusted to 6.0 with HCl. The liquid was again passed first through 0.45 µm and then 0.22 µm Stericups (Millipore, Inc.). The cell-free *F. virguliforme* culture filtrates were used in stem-cutting assays, western blot analysis or stored at -20°C until further use (Li et al., 1999). For analysis of FvTox1 expression, cultures grown for 2, 4, 6, 8, 10, 12, 14 and 16 days were used.

Gel Filtration

Gel filtration of the cell-free Fv culture filtrates was carried out in a ~30 cm long Sephacryl S300HR column (Sigma-Aldrich, St. Louis, MO). The range of separation of this matrix was 10 to 1500 kDa globular proteins. 50 mM Tris-HCl pH 5.5 was used for elution of the proteins. About 3.0 mg proteins were loaded in each run and ~ 1 mL fractions were manually collected. Protein contents were measured in a colorimetric assay based on binding to Bradford dye (Bio-Rad, Hercules, CA).
Stem-Cutting Assay

Seeds of cultivar Williams 82, susceptible to *F. virguliforme*, were grown under light at 25°C in growth chamber for 16 h and in dark at 16°C for 8 h. The light intensity was 200 µmol photons m⁻² sec⁻¹. Three-week old seedlings were cut below the cotyledons and used for bioassays. Cut seedlings were placed into 50 mL Falcon tubes containing 20 mL of the test solutions. Crude cell-free *Fv* culture filtrates, column fractions, or eluted protein samples from polyacrylamide gels were diluted in sterile water for conducting stem-cutting assays. For testing the phytotoxic activity of column fractions, 500 µL eluent from each fraction was diluted to a final volume of 20 mL with sterile water. Water, diluted MSM, and 50 mM Tris-HCl pH 5.5 were used as controls. Symptoms appeared 7-8 days following feeding the *Fv* culture filtrates. The scoring scheme was similar to the one used by Ji et al. (2006). 0, no symptoms; 1, <10% chlorosis; 2, 10 to 20% chlorosis; 3, 20-50% chlorosis; 4, 50-80% chlorosis and necrosis; 5, entire leaf was chlorotic or necrotic.

Proteinase K Treatment

To denature the proteins, the protein pellet was resuspended in 7 M urea, 50 mM Tris-HCl pH 8, and 3 mM dithiothreitol. The protein solution was heated to 60°C for 1 h. Samples were allowed to cool and CaCl₂ was added to a final concentration 5 mM in 50 mM Tris-HCl, pH 7.5. Proteinase K was added to the protein sample to a final concentration of 100 µg mL⁻¹ and then incubated at 50°C for 1 h. To terminate the reaction, phenylmethanesulphonylfluoride (PMSF) was added to a final concentration 5 mM and incubated at 65°C for 15 min.
Protein Fractionation in Native Polyacrylamide Gels

Lyophilized cell-free Fv culture filtrates were dissolved in autoclaved, double-distilled water and then mixed with 2X loading buffer. A 50 µL sample was separated in 12% or 14% native-polyacrylamide gels carrying no sodium dodecyl sulfate. Gels were run at 110 volts for 5 h in a Bio-Rad Protean II system (Bio-Rad, Hercules, CA). The gels were divided into three longitudinal sections (Figure 2). The two outer longitudinal sections of ~3 to 4 cm were fixed, stained and destained to visualize the protein profiles. Stained gel slices were aligned with the large unstained middle longitudinal gel section. The middle unstained gel section was dissected into six horizontal gel slices (Figure 2A) and proteins from each slice were extracted as follows. Gel slices were ground into very small pieces and mixed with water to a final volume of 15 mL in Falcon tubes and vortexed at maximum speed for 2 min. The tubes were stored at -20°C for overnight. Following day, the tubes were thawed and centrifuged at 3,000 rpm for 20 min. The supernatant was filtered through a layer of Whatman No. 1 filter paper and the eluents was diluted in sterile water for stem-cutting assays.

Generation of Anti-FvTox1 Monoclonal Antibodies

The putative FvTox1 protein in native PAGE gel slices was injected into four mice of the Balb/c line at the Iowa State University Hybridoma Facility. After two weeks, another injection was given to boost the antibody production. Two weeks after the second injection, blood was drawn from the mice; serum was separated by using a serum gel clotting activator column (Sarstedt Inc. Newton, NC). Sera were tested for antibody titer, first by ELISA and
then by western blot analyses. The mouse showing the strongest anti-FvTox1 antibody reaction to FvTox1 (Figure 4) was sacrificed and its spleen was dissected out. The splenocytes were fused to mouse myeloma cells (SP2/0) to generate hybridoma cells. The hybridoma clones were tested by conducting ELISA for cross reactivity to FvTox1. ELISA positive clones were tested for cross reactivity to FvTox1 in western blot analyses (Figure 4).

**Western Blot Analyses**

Protein contents were determined by colorimetric assay based on binding to Bradford dye (Bio-Rad, Hercules, CA). Equal amounts of proteins from each sample were separated on denaturing PAGE gels. The gels were blotted onto Optitran nitrocellulose membrane (Midwest scientific, St.Louis, MO). The membranes were hybridized to either anti-FvTox1 monoclonal antibody or anti-His tag antibody (Sigma-Aldrich, St. Louis, MO. Hybridization was detected using goat anti-mouse antibody conjugated to alkaline phosphatase (Bio-Rad, Hercules, CA).

**Immuno-Suppression of the Phytotoxic Activity of Fv Culture Filtrates**

Concentrated cell-free Fv culture filtrates containing about 100 µg proteins were incubated with 500, 300 or 100 µL of anti-FvTox1 monoclonal antibody in 0.6X phosphate buffer saline (PBS), pH 7.4 for 6 h at 4ºC with shaking. Tubes were centrifuged at 4000 g for 3 min. Supernatant was used for the stem-cutting assays.
Construction and Screening of an Expression cDNA Library

Total RNA was extracted from Fv mycelia grown for 10, 11, 12, 13, and 14 days in MSM liquid medium. Poly(A+) RNA was isolated from total RNAs using PolyATract mRNA Isolation System IV (Promega, Madison, WI). An expression F. virguliforme cDNA library for mycelia was constructed in λTriplEx2 (Clontech, Mountain View CA) was provided kindly by William A Moskal, The Institute of Genomic Research. The λTriplEx2 expression library was screened using the anti-FvTox1 7E8 monoclonal antibody according to the Clontech protocol (Mountain View, CA). About 500,000 plaque forming units were screened. The positive plaques were re-screened. By infecting E. coli strain BM25.8 with pure phage particles, λTriplEx2 particles were excised into pTriplEx2 plasmids according to the Clontech protocol (Clontech, Mountain view, CA). E. coli BM25.8 strain carrying individual putative cDNA plasmids was grown in 2 mL SOB medium containing ampicillin at 37°C for overnight with shaking. The following day, 25 mL SOB medium was inoculated with the overnight culture to an OD$_{600}$ of 0.1 and grown at 37°C to 0.4 - 0.6 OD$_{600}$. One mL aliquot from each culture was centrifuged and the pellet was stored at -20°C until further use. To the rest of the culture, IPTG was added to a final concentration of 1 mM and grown at 37°C for 4 h. Aliquots of 1 mL were taken every hour and centrifuged and pellets were stored at −20°C until their uses. Cell pellets were resuspended in 100 µL 20 mM PBS, pH 7 buffer. The mixture was frozen in liquid nitrogen and then thawed at 42°C. Freeze-thaw was repeated four times. The tubes were centrifuged at 13,000 rpm for 10 min at 4°C. The supernatant was transferred into a new Eppendorf and the pellet was resuspended in 100 µL of 2X sodium dodecyl sulfate sample buffer [0.1 M Tris (pH 6.8), 2% sodium dodecyl sulfate, 20% glycerol, 2% β-mercaptoethanol, 0.1% bromophenol blue] and boiled for 5 min
and resolved in a sodium dodecyl sulfate-PAGE gel. The supernatant was mixed with 100 µL of 2X loading buffer and separated in native-PAGE gel and denaturing PAGE gels. Western blot analyses were conducted using all four antiFvTox1 monoclonal antibodies. cDNA inserts of positive pTriplEx2 clones were sequenced.

**Genomic Library Screening**

DNA was isolated from *Fv* mycelia grown for 10, 11, 12, 13 and 14 days in MSM liquid medium using cetyl trimethylammonium bromide (CTAB) method. A genomic library was constructed in lambdaFixII vector (Stratagene, La Jolla, CA). The library was screened using a radiolabeled *FvTox1* cDNA molecule. Purified genomic clones were PCR amplified using primers-specific to the *FvTox1* cDNA molecule (FvTox1F-ATGAAGTCCACATTCACCCTTG, FvTox1R-GCGCTGTGGGTTGCGCACACAGTTG).

**Gel Electrophoresis and DNA Blot Analysis**

Approximately 5 µg DNA was incubated overnight at 37°C with individual restriction enzymes (New England Biolabs, Beverly, MA). The digested DNA samples were electrophoresed on 0.8% agarose gels. The DNA was then transferred onto Zeta-Probe GT membrane (Bio-Rad, Inc., Hercules, CA, USA) using the capillary action of 0.4N NaOH and 1.5M NaCl solution. The filters were neutralized in 100 mM Tris-HCl pH 7.5 for 5 min and washed twice in 2X SSC for 5 min each. The filters were air dried and baked between 2 sheets of Whatman No. 3 MM paper for 2 h at 80°C.

DNA filters were prehybridized in 6x SSC buffer containing 0.5% sodium dodecyl sulfate, 5x Denhardt's solution and denatured salmon sperm DNA (200 µg/m (Sigma-
Aldrich, St. Louis, MO) for 2 h at 45°C. [32P]ATP (PerkinElmer, Waltham, Massachusetts) labeled cDNA molecules were prepared according to (Feinberg and Vogelstein, 1983). Unincorporated nucleotides were removed from the radio-labeled probes using a Sephadex G-50 column (Bio-Rad, Inc., Hercules, CA, USA). The prehybridization buffer was replaced with the purified P32-labeled probe in fresh 6x SSC buffer and hybridized to the DNA filters for 16 h at 45°C. After 16 h, the filers were washed with 6X SSC/0.5% sodium dodecyl sulfate for 30 min at 45°C, then with 3X SSC/0.5% sodium dodecyl sulfate for 45 min at 45°C and finally with 2X SSC/0.5% sodium dodecyl sulfate for 45 min at 45°C. The filters were then wrapped in between two Saran Wrap pieces and exposed to X-ray films overnight at -80°C.

**Sequencing and Analysis of the Fv Genome**

The Fv genomic DNA was sequenced at the DNA facility, Iowa State University (ISU) using a Solexa Genome Analyzer II. Solexa GAII can uncover sequences of billions of DNA bases in each sequence run. A library of 150-200 bp fragments was created and ligated to adapters. The fragments were then bound to the flow cell by hybridizing the fragments to a lawn of oligos complementary to the adapter sequences. Amplification was done to create millions of dense clusters. The clusters were sequenced using a sequencing primer. The sequencing was conducted for 75 cycles and sequence reads of 75 bases were obtained. Sequencing was conducted in two lanes and 20 Fv genome equivalents sequence data were obtained. The Fv genome sequence was searched for FvTox1 sequences using nucleotide blast program ([http://blast.ncbi.nlm.nih.gov/Blast](http://blast.ncbi.nlm.nih.gov/Blast)).
FvTox1 Expression in Insect Cells

Sf21 cells derived from the fall armyworm, Spodoptera frugiperda were maintained in TC-100 insect cell medium (Sigma, St Louis, MO) supplemented with fetal bovine serum (FBS; Gibco-BRL) to a final concentration of 10% and antibiotics (1 U penicillin/mL, 1 mg streptomycin/mL; Sigma, St Louis, MO). The cell cultures were maintained at 28°C as monolayers in screw-capped plastic flasks (Fisher scientific, Pittsburgh, PA). Pro-FvTox1 and mature-FvTox1 were PCR amplified and cloned into the bacmid transfer vector, pFastBac1. Transfer vector carrying either of the FvTox1 genes was used to make recombinant bacmids by following the Bac-to-Bac Baculovirus Expression System protocol (Invitrogen, Carlsbad, CA). Sf21 cells were transfected with the recombinant bacmids to produce recombinant baculoviruses carrying FvTox1 sequences or only empty pFastBac1 vector. Baculovirus expresses recombinant proteins from the polyhedrin promoter. Cells were harvested at 72 h post-infection (p.i.), pelleted, and resuspended in 1X sodium dodecyl sulfate-PAGE sample buffer. Aliquots (50,000 cell equivalents) of the cell extracts were run on 12% sodium dodecyl sulfate polyacrylamide gels. Gels were stained with Coomassie blue G-250 for visualization of proteins.

Leaf Disc Assay

Seeds of various cultivars of soybean were grown under light at 25°C in growth chamber for 16 h and in dark at 16°C for 8 h. The light intensity was 200 µmol photons m⁻² sec⁻¹. Unifoliates were harvested from 17 to 20 d old seedlings and leaf discs were cut out using a 15 ml falcon tube. The recombinant proteins and the culture filtrate were diluted 1:5 (unless otherwise mentioned), using deionized water. These test solutions were added to a 50 ml
Falcon tube and 8 leaf discs were added to each. A pasture pipette was put on the top of the leaf discs to help them dip in the test solution. The leaf discs were vacuum infiltrated, until the solutions were seen infiltrating into the leaf discs. The leaf discs were taken out into the Petri plates and left under light (100-150 µmol photons m⁻²sec⁻¹) conditions. The leaf discs were photographed 4 days following infiltration.

**Mass Spectrometry Analysis**

To confirm the identity of FvTox1 expressed in insect cells, we conducted MS-MS analysis for pro-FvTox1 and the purified toxin from Fv culture filtrates as follows. The stained protein bands from a 12% sodium dodecyl sulfate polyacrylamide gel were precisely cut and subjected to tryptic digestion for MALDI-MS/MS analyses. MALDI-TOF MS/MS analyses were performed using a QSTAR XL quadrupole TOF mass spectrometer (AB/MDS Sciex, Toronto, Canada) equipped with an oMALDI ion source at the ISU Proteomic Facility. All spectra were processed by MASCOT (Matrix Science, London, UK) database search. Peak lists were generated by Analyst QS (AB/MDS Sciex, Toronto, Canada) and were used for MS/MS ion searches.

**N-terminal Sequencing**

Following the transfer of proteins onto a Hybond-P PVDF membrane, membrane was stained with 0.1% Coomassie blue G-250, destained with 50% methanol several times until proteins were visible and background was clear. The membrane was then washed six times with deionized water and the proteins were precisely excised from the PVDF membrane and
sequenced by Edman degradation method in a 494 Precise Protein Sequencer/140C Analyzer (Applied Biosystems, Inc.) at the ISU Protein Facility.

**Measurement of Chlorophyll Contents**

Soybean leaf discs infiltrated with protein preparations were placed individually in Eppendorf tubes and frozen overnight at -80°C. Next day, 1 mL 80% acetone was added to each tube and the tubes were incubated at room temperature in the dark for 5 d. The acetone solution containing chlorophylls was measured for absorbency at 645 nm and 663 nm. The amount of chlorophylls was calculated essentially according to method described by (Arnon, 1949).

**Inoculation of Seedling with *F. virguliforme***

Soybean seeds were grown in a 1:1 mixture of sand and soil containing *F. virguliforme* inoculums grown on sorghum seeds according to (Mueller et al., 2002). Control plants were grown only in sand and soil mixture. The roots from the infected and control plants were collected and used for RNA preparation. Etiolated seedlings were grown in the dark for 5 d at 22°C in coarse vermiculite. Ten etiolated seedlings were placed in a 50 mL Falcon tube containing 20 mL *F. virguliforme* conidial suspensions (2 x 10⁶/mL). Control seedlings were fed with 10 ml water only. Roots were harvested 7 days following infection or treatment with water and stored at -80°C. For protein preparation, the frozen samples were ground in buffer containing 5 mM EDTA, 100 mM Potassium Phosphate, 1% Triton, 10% glycerol and proteinase inhibitor cocktail 1µL/mL (Sigma-Aldrich, St. Louis, MO). The resulting slurry
was centrifuged at 12,000 rpm at 4°C. The supernatant was mixed with 4X sodium dodecyl sulphate loading dye and separated in a 12% polyacrylamide gel. The gel was blotted overnight onto a nitrocellulose membrane (Midscience, St. Louis, MO) and western blot analysis was conducted using anti-FvTox1 7E8 monoclonal antibody.

**RNA Preparation and RT-PCR**

RNAs were prepared using Trizol (Invitrogen, Carlsbad, CA). RNAs were treated with DNase (Invitrogen, Carlsbad, CA) and the first strand cDNAs were synthesized using Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA). cDNAs were used as templates for PCR. The forward primer (FvToxExoF-CACGCTGGAATGGTCTACTTTTG) for PCR was synthesized based on the junction sequence between the 3’end of the first exon and the 5’ end of the second exon. The reverse primer (FvToxExoR-GGGCGCTGAGTGTTGCCATC) was designed based on the junction sequence between the 3’end of the second exon and the 5’ end of the third exon. These primers were expected to amplify a fragment of 173 bp only from cDNAs, not from contaminating genomic DNA.

**Accession Numbers:** Sequence data from this article can be found in the EMBL/GenBank data libraries under accession number(s) FOXG_06271.2 (Fo-Pp); FVEG_04124.2 (Fver-Hp); EEU40946.1 (Nh-Hp); XP 389746.1(Gz-Hp). FvTox1 can be found under accession number X.
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Figure 1. Purification of a phytotoxic protein(s) from cell free Fv culture filtrates by column chromatography. (A) Association of disease severity with protein concentrations in individual column fractions. Optical densities of individual fractions were measured at 595 nm to determine relative protein contents of 1 mL column fractions. SDS symptoms
produced by individual column fractions in stem-cutting assays was scored as follows. 0, no symptoms; 1, < 10% chlorosis; 2, 10 to 20% chlorosis; 3, 20-50% chlorosis; 4, 50-80% chlorosis and necrosis; 5, entire leaf became chlorotic or necrotic. (B) Electrophoresis of column fractions in a 14% native polyacrylamide gel showed association of the disease severity of individual column fractions with a low molecular mass protein (shown by an arrow).

Figure 2. Identification of a protein that showed phytotoxic activity. (A) A 14% native polyacrylamide gel shows protein profile of cell-free Fv culture filtrate. Gel was divided into three longitudinal sections. Two outer sections were stained with Coomassie Brilliant Blue to identify protein profiles. Middle unstained portion of gel was divided into six horizontal fractions as shown in A. Proteins were extracted from each of these six fractions and tested
for their phytotoxic activity in stem-cutting assay. (B) Fractions I through V did not show any phytotoxicity. (C) Fraction VI produced foliar SDS symptoms, when it was fed to cut soybean seedlings.

**Figure 3.** Purified FvTox1 is a small molecular mass protein. (A) Electrophoresis of purified FvTox1 protein in a 12% native polyacrylamide gel. Molecular mass of FvTox1 was estimated to be <10 kDa; (B) Sodium dodecyl sulfate polyacrylamide gel electrophoresis of purified FvTox1. Under denaturing conditions, molecular mass of FvTox1 was ~13.5 kDa.
Figure 4. Generation of anti-FvTox1 antibodies. (A) Anti-FvTox1 polyclonal antibodies. (a), Coomassie Blue stained native gel showing protein profiles of crude cell-free \( F_v \) culture filtrate (CF) and purified column fractions (P) that produced foliar SDS symptoms in stem-cutting assays. (b) Western blot analysis of gel in (a) with mouse anti-FvTox1 polyclonal antibodies showed specific reaction of antibodies to a small molecular mass protein (shown by an arrow). (B) Reactions of anti-FvTox1 monoclonal antibodies to FvTox1. (a), Coomassie Blue stained gel showing protein profiles of cell-free \( F_v \) culture filtrate (CF), gel purified FvTox1 (P). (b) - (e), Western analyses of the protein samples shown in (a) with anti-FvTox1 monoclonal antibodies 2F3 (b), 13F5 (c), 7E8 (d) and 15C2 (e).
Figure 5. FvTox1 is encoded by a single gene.

(A) Sequence of FvTox1 gene encoding FvTox1. TATA box is shown in bold. Start and end of introns are shown by bold capital letters. (B) Low stringency Southern analysis of F. virguliforme DNA revealed that FvTox1 is a single copy gene.
Figure 7. Expression of FvTox1 in an insect cell line. (A) Denaturing-PAGE analysis of expressed FvTox1 proteins. Sf21 cells infected with either empty vector (V), mature-FvTox1 (M) or pro-FvTox1 (P) were harvested 72 h post infection. Equal amounts of whole cell lysates were separated in a 12% sodium dodecyl sulfate-PAGE gel and stained with Coomassie brilliant Blue; ~17 kDa expressed pro-FvTox1 is indicated by an arrow. (B) Western blot analysis of expressed FvTox1 proteins. Protein preparations from whole Sf21 cell lysates carrying empty vector (V), mature-FvTox1 toxin (M) and pro-FvTox1 (P) along with proteins from cell-free Fv culture filtrate (CF) were hybridized to anti-FvTox1 7E8 monoclonal antibody.
Figure 8. Recombinant FvTox1 proteins cause loss of chlorophyll in soybean leaf discs. (A) Loss of chlorophylls in a SDS-susceptible soybean line, Essex infiltrated with protein preparations from Sf21 insect cell lines carrying either the empty pFastBac1 vector (V), pro-FvTox1 (P), mature-FvTox1 (M), or cell-free Fv culture filtrate (CF). (B) Chlorophyll contents in leaf discs of SDS-resistant (SB, SB2859R; MN, Mn-1606SP; 2, 233+RR), SDS-partial resistant (F, Forrest) and SDS-susceptible (H, H2494; S, S03-007CR; 5, 5171RR; and E, Essex) soybean cultivars vacuum infiltrated with protein preparations from Sf21 cells.
carrying either empty pFastBac1 vector (V), mature-FvTox1 (M) or pro-FvTox1 (P) and cell-free Fv culture filtrate (CF). Error Bars represent Standard error (n-1=23).

**Figure 9.** Mature-FvTox1 is much more phytotoxic than pro-FvTox1. (A) Western blot analysis showed approximately 100X higher expression levels of pro-FvTox1 than that of mature-FvTox1 in Sf21 insect cell line (presented in Figure 7). (B) Quantification of chlorophyll contents in leaf discs infiltrated with similar amounts of mature-FvTox1 (M), pro-FvTox1 (P) and FvTox1 from cell-free Fv culture filtrate (CF). E, Essex; H, H2494; SB, SB2895R; MN, Mn-1606SP. Error Bars represent Standard error (n-1=15).
Figure 10. Distinct FvTox1 cleavage sites in an insect cell line and *F. virguliforme*. (A) Western blot analysis of Sf21 insect cells expressing recombinant His-tagged pro-FvTox1 protein. In left panel, whole cell proteins of Sf21 insect cells carrying empty vector (V) or His-tagged pro-FvTox1 (P) were resolved in a 12% sodium dodecyl sulfate -PAGE gel and detected by anti-FvTox1 antibody. In right panel, western blot analysis of His-tagged pro-FvTox1 (P) and His-tagged GmPUB1-1 protein (U) (as a positive control) using anti-His antibody is shown. Note that cleavage of N-terminal His-tag resulted in failure of anti-his antibody to detect His-tagged pro-FvTox1 protein. (B) Location of cleavage sites in pro-FvTox1: site 1 (between two alanine residues) in Sf21 cells and site 2 (between arginine and serine residues) in *F. virguliforme*. (C) FvTox1 was excreted by Sf21 cells into culture medium. V, Sf21 cells infected with empty vector; P, pro-FvTox1; M, mature-FvTox1. Sf21 cell cultures were harvested at 72 h post inoculation. Cell-free Sf21 culture filtrates were used for western blot analysis.
**Supplementary Figure 1.** Phytotoxic activity of crude cell-free $F_v$ culture filtrates was abolished following Proteinase K treatment. (A) Sodium dodecyl sulfate PAGE of protein samples prepared from column fractions of cell-free $F_v$ culture filtrates. Fraction numbers are shown at top of gel. CF, Crude cell-free $F_v$ culture filtrates. (B) Sodium dodecyl sulfate PAGE of column fractions obtained from Proteinase K-treated crude cell-free $F_v$ culture filtrates. None of these fractions produced any foliar SDS symptoms in stem-cutting assay.
Supplementary Figure 2. Immuno-suppression of FvTox1 from cell-free Fv culture filtrate by anti-FvTox1 antibody resulted in suppression of foliar SDS development. FvTox1 was precipitated using different amounts of anti-FvTox1 antibody (7E8). Resulting supernatants were fed to cut soybean seedlings. (A) Increased levels of SDS foliar symptoms developed with reduced amount of antibody usages in the immune-precipitation experiments. (B) Increased foliar SDS with reduced usages of antibody in immune-precipitation experiments. SDS symptoms produced by individual treatments in stem-cutting assays were scored as follows. 0, no symptoms; 1, <10% chlorosis; 2, 10 to 20% chlorosis; 3, 20-50% chlorosis; 4, 50-80% chlorosis and necrosis; 5, entire leaf became chlorotic or necrotic. Error bars represent Standard error (n-1=7).
Supplementary Figure 3. Alignment of FvTox1 with its homologous sequences. Fo-Pp, *Fusarium oxysporum* protein (FOXG_06271.2); Fver-Hp, *Fusarium verticillioides* protein (FVEG_04124.2); Nh-Hp, *Nectria haematococca* protein (EEU40946.1); Gz-Hp, *Gibberella zeae* (XP 389746.1). "**", identical residues; "*:", conserved substitutions between similar residues; "\.", semi-conserved substitutions between similar residues. The percent identity of FvTox1 to FOXG_06271.2, FVEG_04124.2, EEU40946.1, XP 389746.1 is 67, 60, 91 and 59, respectively.
Supplementary Figure 4. Loss of chlorophyll in leaf discs of cultivar Essex (SDS-susceptible) and Forrest (carries partial SDS resistance) following infiltration with FvTox1. V, proteins from insect cells carrying empty vector; P, pro-FvTox1 expressed in insect cells; M, mature-FvTox1 expressed in insect cells; CF, cell-free Fv culture filtrate.
Supplementary Figure 5. SDS-susceptible but not the SDS-resistant lines were sensitive to expressed mature-FvTox1 and cell-free \textit{Fv} culture filtrates. Pictures were taken four days after infiltration with either mature-FvTox1 or cell-free \textit{Fv} culture filtrates.
**Supplementary Figure 6.** Light is essential for mature-FvTox1-mediated necrosis in soybean leaves. Leaf discs from susceptible cultivar Essex were infiltrated with mature-FvTox1 expressed in an insect cell line, and subjected to either light or dark condition for three days.
CHAPTER 3 EXPRESSION OF A FUNCTIONAL SINGLE CHAIN FRAGMENT ANTIBODY AGAINST A PHYTOTOXIN REDUCED THE INCIDENCE OF A FUNGAL DISEASE IN STABLE TRANSGENIC SOYBEAN PLANTS

Hargeet K. Brar and Madan K. Bhattacharyya

Abstract

A proteinaceous *Fusarium virguliforme* toxin, FvTox1, has been shown to be a major cause of foliar sudden death syndrome (SDS) in soybean. We have created two single-chain variable fragment (scFv) antibody genes, *Anti-FvTox1-1* and *Anti-FvTox1-2* by using the hybridoma cell line that expresses an anti-FvTox1 antibody. *Escherichia coli* expressed Anti-FvTox1 scFv antibodies successfully interacted to FvTox1. Anti-FvTox1 antibody expressed in transformed soybean roots specifically bound to the putative antigenic site of FvTox1. In stable transgenic soybean plants, expression of Anti-FvTox1-1 antibody resulted in enhanced foliar SDS resistance as compared to the non-transgenic control. These results suggested that (i) FvTox1 is a major pathogenicity factor for foliar SDS development and (ii) expression of an Anti-FvTox1 scFv antibody is a suitable biotechnology approach in fighting this serious soybean disease. Our study suggests that expression of plant antibodies should most likely generate resistance against those plant diseases that are induced by pathogen toxins.
**Introduction**

A major distinction between plants and animals is the ability of animals but not plants to produce antibodies against foreign antigens including that of (i) pathogenic microorganisms, (ii) pollen grains and environmental pollutants that causes allergic reactions. Plants on the other hand use a battery of both preformed and active defense compounds to fight invading pathogens (Bonas and Lahaye, 2002). Transgenic plants can however assemble mammalian antibodies correctly. In early studies, genes encoding heavy and light chains of the antibody molecules were either expressed independently in individual transgenic plants and then assembled in the hybrids or co-expressed in the same transgenic plant to obtain the assembled antibody molecules (Hiatt et al., 1989; De Neve et al., 1993). However, due to the complexity in cloning the entire antibody molecules and most importantly differences in post translational modifications between plant and mammals, this initial technology of plant antibody production did not become a method of choice. Subsequently, expression of only the variable regions of the antibody molecule involved in antigen binding was attempted (Bird et al., 1988). These include (i) fragment antigen binding (Fab) antibodies consisting of both variable heavy and variable light chains (Supplementary Fig. 1), (ii) single-domain antibody expressing only the heavy chain regions and (iii) single chain variable fragment (scFv) antibody expressing only the variable regions of one heavy and one light chain. These smaller antibody molecules suffer less from folding and post translation modification when they are expressed in plants and became methods of choice. Other advantage of small antibody molecules with little demand for folding is that they can be targeted to any sub-cellular compartments as well as to extracellular matrix.
Due to their small sizes and the ease in their assembly following expression, scFv antibodies have become the molecules of preference for antibody production in a wide range of organisms. Variable light and heavy chain regions (Supplementary Fig. 1a) of antibody molecules are the basis of generating scFv antibody genes, which are successfully expressed in foreign hosts including plants and bacteria without losing their antigen binding properties (Better et al., 1988; Huston et al., 1988; Chaudhary et al., 1990; Yuan et al., 2000; Galeffi et al., 2002). The scFv antibodies have been successfully expressed in plants (Hiatt et al., 1989; Yuan et al., 2000; Galeffi et al., 2002). In the synthetic scFv antibody genes, both variable heavy (VH) chain and variable light (VL) chain fragments are joined by a polylinker containing glycine and serine residues (Bird et al., 1988) (Supplementary Fig. 1b). Recent studies have shown that addition of the KDEL sequence to the C-terminus contributes towards retention of the scFv molecules in endoplasmic reticulum and its accumulation in plant cytosol (Schouten et al., 1997; De Jaeger et al., 1999). This C-terminal KDEL also helps to stabilize the scFv in the plant cytosol (Ko et al., 2003).

The scFv antibodies have been generated against various toxins. For example, a scFv was synthesized against a mycotoxin, deoxynivalenol, and shown to be functional when expressed in *E. coli* (Choi et al., 2004). An *E. coli* expressed scFv against the cassiicolin toxin was shown to reduce the necrotic lesion formation, when applied as droplets on the abaxial surface of the detached leaves of rubber tree (Sunderasan et al., 2009). A scFv antibody against zearalenone toxin, expressed in transgenic *Arabidopsis* plants, bound to the toxin in ELISA (Yuan et al., 2000) Although several studies established the feasibility of production of scFv antibodies against viruses in plants (Galeffi et al., 2002; Galeffi et al.,
to our knowledge scFv antibodies have not been shown to suppress the phytotoxic effects of pathogen toxins in stable transgenic plants.

Sudden death syndrome (SDS) is a serious, emerging disease of soybean in the United States. The estimated annual crop losses from SDS are valued over 300 million dollars (Wrather and Koenning, 2006). Resistance to SDS is partial. It is encoded by more than 14 QTL (Kazi et al., 2008). Single genes encoding SDS resistance have not yet been identified. SDS is caused by a soil borne fungus, *Fusarium virguliforme*, earlier known as *F. solani* f. sp. *glycines* (Rupe, 1989). The pathogen has never been isolated from diseased foliar tissues. A toxin, FvTox1, released by the pathogen to the infected roots produces the foliar SDS. FvTox1 has recently been isolated. It is a 13.5 kDa acidic protein encoded by a single gene (Brar et al., 2010).

Mouse monoclonal antibodies raised against FvTox1 have been shown to be specific to FvTox1 and effective in suppressing the phytotoxic activity of FvTox1 that causes foliar SDS (Brar et al., 2010). We therefore used the anti-FvTox1 monoclonal antibody, 7E8, to create an anti-FvTox1 scFv antibody. A PCR approach was used to clone two *Anti-FvTox1* scFv antibody genes that were analyzed for their FvTox1-binding properties initially by expressing in *E. coli* and then in *Agrobacterium rhizogenes*-induced transformed soybean roots (Eswarakumar et al., 1997). Anti-FvTox1-1 antibody was able to significantly reduce the development of foliar SDS in stable transgenic soybean plants as compared to the non-transgenic control. The results presented here not only establish for the first time that the plant antibody can fight a toxin-induced plant disease, but also that FvTox1 is a major virulence factor for foliar SDS development in soybean. This biotechnology approach should be applicable to fighting other plant diseases induced by toxins.
Results

Cloning and expression of Anti-FvTox1 antibody genes

The cDNAs generated from the hybridoma cell line expressing anti-FvTox1 monoclonal antibody, 7E8, were used in PCR amplification of variable heavy and variable light antibody fragments. One fragment, 8-1 (468 bp), specific to heavy chain variable (VH) region and two fragments, 4-2 (399 bp) and 6-1 (270 bp), specific to the light chain variable (VL) region were PCR amplified (Supplementary Fig. 1c) and cloned. Each of the three DNA fragments showed high similarity to previously cloned variable regions of other antibodies (Supplementary Fig. 2 – 4). We used the VH and both VL fragments to construct two scFv antibody genes, *Anti-FvTox1-1* (8-1/4-2) and *Anti-FvTox1-2* (8-1/6-1) by joining the VH fragment to individual VL fragments with a flexible polylinker (Gly4Ser)3 sequence (Supplementary Fig. 1d).

The Abysis database (http://www.bioinf.org.uk/abysis/index.html) was used to search the complementarity determining regions (CDRs) of the synthetic Anti-FvTox1 scFv antibodies. As expected, 3 CDRs were found in VH chain 8-1 and VL chain, 4-2 (Supplementary Fig. 5). In the second VL chain, 6-1, only 2 CDRs were found because it was a truncated VL fragment. Two cysteine residues at positions H22 and H92 were conserved in VH fragment, 8-1. In the light chain, 4-2, the cysteine residue was conserved only at position L88 and a tyrosine residue replaced the cysteine residue at position L23. The VL chain, 6-1 did not carry any cysteine residues at conserved L23 and L88 positions (Supplementary Fig. 5). *Anti-FvTox1-1* and *Anti-FvTox1-2* were cloned into the *E. coli* expression vector pRSET (Invitrogen, Carlsbad, CA). The *E. coli* total proteins were separated in denaturing
polyacrylamide gels. The western blot analysis conducted using the goat anti-Xpress antibody (Invitrogen, Carlsbad, CA) revealed that both Anti-FvTox1-1 and Anti-FvTox1-2 scFv antibodies were expressed in *E. coli* (Supplementary Fig. 6). Expressed fusion scFv antibodies containing N-terminal His and Xpress tags were predicted to be slightly larger than 30 kDa (Supplementary Fig. 6).

**E. coli expressed Anti-FvTox1 scFv antibodies interacted with FvTox1.**

The *E. coli* expressed antibody proteins were evaluated for their binding abilities to FvTox1 by conducting western blot analyses. Xpress epitope fused to N-termini of both antibodies was used to monitor the interaction between FvTox1 and *E. coli* expressed Anti-FvTox1 scFv antibodies. Protein preparations from (i) *F. virguliforme* (*Fv*) culture filtrates carrying FvTox1 and (ii) a baculovirus infected SF21 insect cell line expressing FvTox1 were separated on a 12% denaturing PAGE gel and then transferred onto nitrocellulose membrane. The membranes were hybridized to the preparations of total *E. coli* proteins containing either of the expressed Anti-FvTox1 scFv antibodies or an expressed soybean protein as the negative control. Binding of Anti-FvTox1 scFv antibodies to FvTox1 was detected using an anti-Xpress antibody (Invitrogen, Carlsbad, CA). Western blot data suggested that both Anti-FvTox1-1 and Anti-FvTox1-2 scFv antibodies interacted to FvTox1 (Fig. 1).

**Plant expressed Anti-FvTox1 scFv antibodies specifically interacted to the FvTox1 region containing the antigenic site.**

To express the Anti-FvTox1 scFv antibodies, both *Anti-FvTox1-1* and *Anti-FvTox1-2* were fused to a DNA molecule encoding KDEL at either their 5’- or 3’-end and cloned into the
binary vector pISUAgron5 (N.N. Narayanan and M.K. Bhattacharyya, unpublished). *Agrobacterium rhizogenes* carrying individual binary vector construct was used to generate hairy roots transformed with *Anti-FvTox1* antibody genes (Supplementary Fig. 7a). The expression of *Anti-FvTox1* antibody genes in the transformed roots was confirmed by RT-PCR and western blot analyses (Supplementary Figs. 7c, 7d and 7e).

The proteins extracted from the transgenic roots were tested for their binding properties to FvTox1 of *F. virguliforme* culture filtrates and to the recombinant FvTox1 protein expressed in the baculovirus infected Sf21 insect cell line. Both scFv antibodies carrying the KDEL tag at their C-termini and Anti-FvTox1-2 carrying the tag at its N-terminus were able to bind FvTox1 (Supplementary Fig. 8d-e). Although both western blot and RT-PCR data suggested that the Anti-FvTox1-1 antibody with KDEL fused to its N-terminus was expressed in transformed soybean hairy roots (Supplementary Fig. 7), it failed to interact with FvTox1 (Supplementary Fig. 8c). These results suggested that KDEL at the N-terminus of Anti-FvTox1-1 antibody most likely blocked the binding activity of the antibody to FvTox1.

To determine if Anti-FvTox1 scFv antibodies interacted to the same FvTox1 antigenic site recognized by the anti-FvTox1 7E8 monoclonal antibody, truncated *FvTox1* molecules were expressed in *E. coli* and separated in a denaturing PAGE gel. Western blot analysis of the truncated forms of FvTox1 suggested that anti-FvTox1 7E8 antibody recognized an epitope located in a region between 222 and 360 bp of *FvTox1* (Fig. 2). Both Anti-FvTox1-1 and Anti-FvTox1-2 scFv antibodies interacted to this region of FvTox1 suggesting that Anti-FvTox1 scFv antibodies retained the FvTox1-binding specificities of the anti-FvTox1 7E8 monoclonal antibody (Figs. 2c and 2e-g).
Anti-FvTox1-1 scFv antibody reduced foliar SDS development in stable transgenic soybean plants.

To determine the utility of a functional Anti-FvTox1 gene, the soybean cultivar, Williams 82 was transformed with the Anti-FvTox1-1 antibody gene carrying KDEL tag at its C-terminus. The progenies of a transformant were analyzed for their tolerance to foliar SDS. Following F. virguliforme infection, a significant reduction in the extent of symptom development was observed among most of the progenies of the transformant as compared to the non-transgenic Williams 82 control plants (Fig. 3; Supplementary Fig. 9). The average disease score for transgenic plants was significantly lower than that of the wild type Williams 82 control plants (Fig. 3e). The average root length, stem length and fresh weight were significantly higher in transgenic plants as compared to that in non-transgenic control plants (Fig. 3f).

We investigated if the transgenic plants were more tolerant to FvTox1 than the control plants. SDS symptom development was recorded following feeding of transgenic and non-transgenic plants with F. virguliforme (Fv) culture filtrates and results are presented in Fig. 4. The transgenic plants expressing Anti-FvTox1-1 showed enhanced tolerance to FvTox1 as compared to the non-transgenic control plants (Figs. 4a-d). Higher chlorophyll contents and lower foliar SDS disease scores were recorded for the transgenic plants as compared to the non-transgenic Williams 82 control plants (Fig. 4e). Non-transgenic plants used in this investigation went through the same transformation steps that were used to generate the transgenic soybean plant carrying the Anti-FvTox1-1 transgene.

We investigated if enhanced tolerance of transgenic progenies was due to expression of Anti-FvTox1-1 scFv antibody from a particular Anti-FvTox1-1 transgene copy. Among eight
progenies of the transformant, one was highly sensitive to the Fv culture filtrates (Fig. 5a). Western blot data suggested that Anti-FvTox1-1 antibody protein was accumulated in toxin-tolerant transgenic plants. Anti-FvTox1-1 antibody was not detected in the FvTox1-sensitive progeny (Fig. 5b). Southern blot analysis suggested that enhanced FvTox1-tolerance was associated with the integration of an Anti-FvTox1-1 transgene copy (Fig. 5c).

**Anti-FvTox1-1 scFv antibody accumulated mostly in the chloroplasts and cytoplasm**

The FvTox1-tolerant transgenic progeny plants showing accumulation of Anti-FvTox1-1 were used for determining the sub-cellular locations of the scFv antibody accumulation by conducting transmission electron microscopy. Anti-FvTox1-1 was localized to cytoplasm and chloroplasts by anti-KDEL primary antibody and gold conjugated goat anti-mouse IgG secondary antibody (Fig. 6).

**Discussion**

SDS is an emerging soybean disease in the United States. It was first reported in 1971 in Arkansas (Hirrel, 1987). Now the disease has been documented in all soybean growing areas of the United States (Roy et al., 1997; Wrather and Koenning, 2006; Ziems et al., 2006). SDS resistance is partial. Fourteen QTL have been shown to associate with SDS resistance (Kazi et al., 2008). Monogenic SDS resistance has not yet been identified. SDS resistance conferred by QTL is not always sufficient to control this disease. Thus, it evident that novel SDS resistance generated through biotechnological approaches is urgently needed to fight this serious disease.
SDS is caused by the fungal pathogen, *F. virguliforme*. It is a root pathogen and has never been isolated from the above-ground diseased tissues (Roy et al., 1989; Rupe, 1989). *F. virguliforme* releases at least one known proteinaceous toxin into the soybean roots. The toxin travels through the vascular tissues to the aerial green tissues, where it causes the leaf scorch, the foliar SDS symptoms (Li et al., 1999; Brar et al., 2010). The proteinaceous toxin FvTox1 is encoded by a single gene (Brar et al., 2010). Here we have established the possible application of an Anti-FvTox1 scFv antibody in reducing the phytotoxic affect of the toxin in stable transgenic soybean plants.

Hybridoma cell line producing the monoclonal anti-FvTox1 antibody, 7E8 was used to isolate VH and VL regions the antibody molecule. This antibody was used in cloning FvTox1 from the pathogen (Brar et al., 2010). The isolated antibody fragments showed high similarities to the previously characterized variable fragments of other antibody molecules (Supplementary Figs. 2-4). Two cysteine residues, at positions H22 and H92 (Kabat et al., 1991), were conserved in the VH chain, 8-1. However, cysteine residues of the VL chains, 4-2 and 6-1, were not completely conserved. In VL chain 4-2, a cysteine residue was found at position L88, but a tyrosine residue instead of a cysteine residue at position L23. VL 6-1 lacks cysteine residues at both positions, L23 and L88. Disulphide bonds between cysteine residues play an important role in correct folding and stabilization of immunoglobin structures. scFv antibodies lacking the conserved cysteine residues in VL fragments had been shown to retain their functionality (Proba et al., 1997; Proba et al., 1998). We have shown that although neither Anti-FvTox1-1(8-1/4-2) nor Anti-FvTox1-2 (8-1/6-1) carries conserved cysteine residues in the VL fragment, both antibodies are functional. VH fragments have been shown to play a major role in binding antigens because of the large
diversity offered by a highly variable CDR3 region of this molecule (Xu and Davis, 2000). VH regions alone have been shown to act as functional antibodies in several studies (Jobling et al., 2003; Rajabi-Memari et al., 2006; Bouaziz et al., 2009). In Anti-FvTox1-2, most likely VH (8-1) fragment alone was capable of binding to FvTox1.

In SDS, the major destruction by the pathogen is accomplished through the foliar SDS or leaf scorch. Defoliation resulting from the phytotoxic actions of toxin(s) produced by F. virguliforme makes the soybean roots even more susceptible to the pathogen. Light is essential for foliar SDS development by the toxin (Ji et al., 2006; Brar et al., 2010). FvTox1 has been localized to chloroplasts (Brar et al., 2010). These results suggest that interruption of photosynthesis by FvTox1 may play a major role in foliar SDS development in soybean. Through immuno-gold electron microscopy we have shown that Anti-FvTox1-1 antibody was accumulated in cytoplasm and chloroplasts of the transgenic soybean plants. Reduced symptom development and accumulation of the antibody in chloroplasts and cytoplasm of transgenic plants indicated that most likely FvTox1 was bound by Anti-FvTox1-1 during its entrance to chloroplasts through the cytoplasm.

Anti-FvTox1-1 scFv antibody enhanced the tolerance of foliage to FvTox1 in the transgenic as compared to the non-transgenic soybean plants (Figs. 4-5). Our study of stable transgenic soybean plants carrying the Anti-FvTox1-1 antibody gene (Figs. 3-5) thus established that (1) FvTox1 is a major pathogenicity factor for foliar SDS development, and (2) expression of plant antibody against FvTox1 can be further explored in reducing or eliminating the foliar SDS component of SDS in soybean. The transgenic plants did not show any detectible abnormality and set seeds normally. This study thus set the stage for
engineering soybean with targeted expression of Anti-FvTox1 under the regulation of a leaf-specific promoter to supplement the tolerance encoded by a large number of QTL.

The expression of scFv antibodies in plants has been shown to protect plants from viral attack (Tavladoraki et al., 1993; Cervera et al., 2010). These antibodies were directed against the viral coat proteins. Foliar diseases, such as foliar SDS in soybean, caused by toxins are difficult to control with either conventional chemical protectants or through engineering defense response pathways. Our study is the first example to demonstrate that expression of a scFv antibody against a fungal toxin can protect a plant species from the phytotoxic affects of a pathogen toxin; and thus, lays the foundation for engineering crop plants against toxin-induced diseases such as tan spot disease of wheat (Ballance et al., 1989; Tomas et al., 1990; Tuori et al., 1995; Zhang et al., 1997), leaf and glume blotch disease in wheat (Lui et al., 2004; Friesen et al., 2006; Friesen et al., 2007; Friesen et al., 2008; Abeysekara et al., 2009), Verticillium wilt disease in cotton (Palmer et al., 2005) and canker stain disease of plane tree (Platanus acerifolia) (Pazzagli et al., 1999).

**Material and methods**

**Syntheses of Anti-FvTox1 antibody genes**

Total RNAs were extracted from a hybridoma cell line expressing the anti-FvTox1 7E8 (Brar et al., 2010) monoclonal antibody using Trizol reagent (Invitrogen, Carlsbad, CA) as per manufacturer’s instructions. Reverse transcription was carried out to generate the first strand cDNA and then PCR was conducted using universal degenerated primers to amplify the VH, 8-1 (Deg CH1 and DegVH1; Supplementary Table 1), VL, 4-2 (DegCk1 and DegVk1) and VL, 6-1 (DegCk1 and DegVk4; Supplementary Table 1) DNA fragments (Eswarakumar et
A PCR thermal cycling (94°C for 2 min, 72°C for 4 min, repeated 7 times) was used to join the individual PCR fragments of variable heavy chain (VH) and variable light chain (VL) using a DNA sequence that encoded the amino acid linker, (Gly4Ser)3. The scFv fragments were cloned into *E. coli* expression vector, pRSET (Invitrogen, Carlsbad, CA) as an *Xho*I and *HindIII* fragment.

**Expression of Anti-FvTox1 scFv antibodies**

*E. coli* BL21 (DE3) pLysS cells were transformed with Anti-FvTox1 scFv/pRSET constructs and grown overnight at 30°C in 2 mL SOB media containing ampicillin (50 µg/mL) and chloramphenicol (35 µg/mL). The overnight culture was diluted to an OD600 of 0.1 and again incubated at 30°C until the culture reached an OD600 of 0.4-0.6. IPTG was added to a final concentration of 1 mM and the cells were allowed to grow for another 5 h. The cells were pelleted and dissolved in 20 mM phosphate buffer at pH 7. The cells were frozen in liquid nitrogen and then thawed at 42°C for 4 times. The culture was then centrifuged at 14,000 g for 10 min at 4°C. The supernatant was mixed with 2X SDS loading dye (125 mM Tris-HCl pH 6.8, 4% w/v sodium dodecyl sulfate, 20% v/v glycerol, 2% v/v β-mercaptoethanol, 0.001% w/v bromophenol blue), and separated on a denaturing PAGE gel. The gel was blotted on Optitran nitrocellulose membrane (Whatman Inc., Kent, UK) and used for western blot analysis. After blocking the membrane with 5% dry milk powder solution in 1X PBS (phosphate buffered saline) at pH 7.5, the mouse anti-Xpress tag antibody (Invitrogen, Carlsbad, CA) and then goat anti-mouse secondary antibody conjugated to alkaline phosphate (Bio-Rad, Hercules, CA) were used to conduct western blot analysis. The binding
of secondary antibody was detected using the AP Conjugate Substrate Kit (Bio-Rad, Hercules, CA).

**Binding of *E. coli* expressed Anti-FvTox1 scFv antibodies to FvTox1**

FvTox1 was cloned in the pFastBac1 vector to mediate its baculovirus mediated expression in the SF21 insect cell line (Invitrogen, Carlsbad, CA). For assaying the binding activity of *E. coli* expressed scFv antibodies, (i) baculovirus expressed FvTox1, (ii) protein preparation from *Fv* culture filtrates containing FvTox1 and (iii) proteins from the insect cell line infected with baculovirus carrying the empty pFastBac1 vector were separated on denaturing PAGE gels. The water soluble protein extracts from *E. coli* expressing Anti-FvTox1 scFv antibodies were diluted 1:10 in 1X PBS (pH 7.5) and used as the primary antibodies. The blots were then incubated with anti-Xpress antibody (Invitrogen, Carlsbad, CA). The goat anti-mouse antibody conjugated to AP (Bio-Rad, Hercules, CA) was used as the secondary antibody. The western blots were developed using the AP Conjugate Substrate Kit (Bio-Rad, Hercules, CA).

**Plant expression vector construction**

The binary vector pISUAgron5 (modified pTF101.1, a derivative of pPZP binary vector (Paz et al., 2004)) was used to develop fusion genes for constitutive expression of Anti-FvTox1 antibody genes under the regulation of the CaMV 35S promoter. Primers (For N-terminal fusion, 8-1 F-KDEL and 4-2R/6-1R; for C-terminal fusion 8-1F and 4-2 R KDEL/6-1 R KDEL; Supplementary Table 1) were used to fuse the four amino acid KDEL-tag in frame with the antibodies at their either N- or C-termini. The binary vector plasmids were
transformed into *Agrobacterium rhizogenes* K599 by freeze thaw method (Holsters et al., 1978).

**Transformation of soybean cotyledons**

Soybean cultivar ‘Williams 82’ was grown in coarse vermiculite at 23°C and a 16 h light (200 μmol photons m⁻² sec⁻¹) period. After 7 days, the cotyledons from the seedlings were harvested, and surface sterilized with alcohol wipes (North Safety Products, Cranston, RI) (Subramanian et al., 2005). A 200 μL pipette tip was used to make a round circular wound at the center of each cotyledon. The cotyledons were again surface sterilized with alcohol wipes. The wounded cotyledons were infected with *A. rhizogenes* culture resuspended in 10 mM MgSO₄. The Petri plates were sealed with parafilm and incubated at 22°C for 12 h under 150 μEs light intensity and 12 h in dark.

**Binding of Anti-FvTox1 scFv antibodies expressed in transformed roots**

The transgenic roots generated 2 weeks following *A. rhizogenes* infection were harvested and frozen in liquid nitrogen. The root samples were grounded in liquid nitrogen and used for protein, DNA and RNA isolation. For protein preparation, the tissue powder was extracted in phosphate buffer (5 mM EDTA, 100 mM Potassium Phosphate, 1% Triton, 10% glycerol and proteinase inhibitor cocktail 1 μl/mL) and centrifuged at a 14,000 g and at 4°C. The supernatant was used as the crude AntiFvTox1 primary antibody to determine its binding activity to FvTox1 on nitrocellulose membranes in western blot analysis. The mouse anti-KDEL monoclonal antibody (Stressgen, Ann Arbor, MI) was used to detect the binding of expressed Anti-FvTox1 scFv antibodies to FvTox1.
RNA preparation and RT-PCR analyses of Anti-FvTox1 antibody genes

RNA was prepared using Trizol reagent (Invitrogen, Carlsbad, CA). RNA was treated with DNase (Invitrogen, Carlsbad, CA) to eliminate any PCR amplification from contaminating DNA. The first strand cDNAs were synthesized using Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA). cDNAs were used as templates for PCR. RT-PCR was conducted using the forward primer of the Anti-FvTox1 antibody genes (8-1F) and the reverse primer from the pISUAgron5 vector (PolyATailR; Supplementary Table 1). The PCR amplified products were separated on agarose gels.

Deletion analysis of FvTox1

PCR primers were used to create truncated versions of FvTox1 (Forward-FvTox1F and Reverse, R3/R4/R7/FvTox1R; Supplementary Table 1). The truncated versions were cloned into E. coli expression vector pRSET (Invitrogen, Carlsbad, CA). The truncated FvTox1 proteins were expressed by following a protocol for expression of Anti-FvTox1 scFv antibodies in E. coli. For separation on denaturing PAGE gels, the cell pellets were dissolved in 2X SDS loading dye (125 mM Tris-HCl pH 6.8, 4% w/v sodium dodecyl sulfate, 20% v/v glycerol, 2% v/v β-mercaptoethanol, 0.001% w/v bromophenol blue). For western blot analyses, Anti-FvTox1 7E8 monoclonal antibody was diluted to 1:500 in 1X PBS.
Infection of soybean seedlings with *Fusarium virguliforme*

Soybean seeds were grown in a 1:1 mixture of sand and soil containing *F. virguliforme* inocula grown on sorghum seeds according to ((Mueller et al., 2002). Data was collected 5 weeks after inoculation.

**Stem-cut assay**

Seeds of the ‘Williams 82’ plants that went through the transformation procedure but did not contain any transgenes, and that of the transgenic Williams 82 plants carrying the *Anti-FvTox1-1* transgene were grown in growth chambers at 25°C for 16 h under light (300 µE) and at 16°C for 8 h in dark. Three-week old seedlings were cut below the cotyledons and used for bioassays. Cut seedlings were placed in 50 mL Falcon tubes containing 25 mL of crude cell-free *Fv* culture filtrates that were diluted in sterile water (1:24) (Li et al., 1999). Symptoms started to appear 8 days following feeding the cell-free *Fv* culture filtrates. The scoring scheme was similar to the one used in earlier study (Ji et al., 2006).

**Measurement of chlorophyll contents**

Soybean leaf discs of approximately 1 cm² size were excised from soybean leaves with or without foliar SDS symptoms and individually placed in Eppendorf tubes and frozen overnight at -80°C. Next day, 1 mL of 80% acetone was added to each tube and the tubes were incubated at room temperature in dark for 5 days. The acetone solution containing chlorophylls was measured for absorbency at 645 nm and 663 nm. The amount of
chlorophylls was calculated essentially according to a method described earlier (Arnon, 1949).

**Western blot analyses**

Protein contents of all samples were determined by colorimetric assay based on binding to Bradford dye (Bio-Rad, Hercules, CA). Equal amounts of proteins from each sample were separated on denaturing PAGE gels. The gels were blotted onto Optitran nitrocellulose membrane (Midwest scientific, St. Louis, MO). The membranes were hybridized to either anti-FvTox1 monoclonal antibody 7E8 or *E. coli* protein extracts containing Anti-FvTox1 scFv antibodies, protein extracts of (i) transformed roots induced by *A. rhizogenes*-mediated transformation or (ii) transgenic plants generated by *A. tumefaciens*-mediated transformation (Paz et al., 2004). Hybridization of mouse monoclonal anti-FvTox1 antibody 7E8 to FvTox1 was detected using goat anti-mouse antibody conjugated to alkaline phosphatase (Bio-Rad, Hercules, CA) and that of Anti-FvTox1 scFv antibodies from *E. coli* by anti-Xpress antibody (Invitrogen, Carlsbad, CA) and then by goat anti-mouse secondary antibody conjugated to alkaline phosphatase (Bio-Rad, Hercules, CA), and that of Anti-FvTox1 scFv antibodies from transformed soybean tissues to the toxin was detected with anti-KDEL antibody (Stressgen, Ann Arbor, MI) followed by goat anti-mouse secondary antibody conjugated to alkaline phosphatase (Bio-Rad, Hercules, CA).
**DNA preparation and Southern blot analysis**

DNA was prepared using a modified CTAB (cetyl-trimethyl ammonium bromide) method (Cullings, 1992). For Southern blot analyses (Southern, 1975), 10 µg DNA of each transgenic plant was incubated overnight at 37°C with EcoRI restriction endonuclease (New England Biolabs, Beverly, MA). The digested DNA samples were separated on 0.8% agarose gels for overnight at 30 volts. The DNA was then transferred onto Zeta-Probe GT Nylon membrane (Bio-Rad, Inc., Hercules, CA) using the capillary action of 0.4N NaOH and 1.5M NaCl solution. The filters were neutralized with 100 mM Tris-HCl pH 7.5 for 5 min and washed twice with 2X SSC for 5 min each. The Southern blot analysis was conducted according to the previously described protocol (Brar et al., 2010). A 32P labeled probe was prepared for the VH chain, 8-1. Hybridization and washing were conducted at 65°C. The filters were then exposed to X-ray films for overnight at -80°C.

**Microscopy**

For transmission electron microscopy (TEM) tissues were sectioned and then fixed with 2% paraformaldehyde (w/v) and 0.1% glutaraldehyde (w/v) in 0.1M Cacodylate buffer pH 7.5 for 2 h at 4°C. Samples were rinsed with dH2O (this and all subsequent procedures except for polymerization were carried out at room temperature). The samples were dehydrated in a graded ethanol series, and infiltrated and embedded using LR White resin (Electron Microscopy Sciences, Ft. Washington, PA). Resin blocks were polymerized for 48 h at 4°C under UV light. Thick and ultrathin sections were prepared using a Reichert UC6 ultramicrotome (Leeds Precision Instruments, Minneapolis, MN) and collected onto formvar coated nickel grids. The grids were blocked for non-specific binding using the incubation
buffer (phosphate buffered saline with 2% BSA-c and 0.01% fish gel) for 30 min at room temp. Then grids were exposed to a 1:500 dilution of anti-KDEL antibody (Stressgen, Ann Arbor, MI) for 3 h at 37°C. The control grids were placed into fresh incubation buffer for 3 h at 37°C. All grids were washed 5 times, 5 min each with incubation buffer and then exposed to a 1:30 dilution of 10 nm gold conjugated goat anti-mouse IgG secondary antibody for 2 h at room temp. All grids were washed with deionized water 5 times, for 5 min each step and then stained with 1% uranyl acetate in deionized water for no more than 5 min. All grids were stream washed with deionized water and dried prior to imaging. Images were captured using a JEOL 2100 scanning and transmission electron microscope (Japan Electron Optic Laboratories, Peabody, MA).

Acknowledgements

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**Supplementary Table 1.** List of primers used in this study

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Figure 1 Binding of *E. coli* expressed Anti-FvTox1 scFv proteins to FvTox1. (a) A protein gel (sodium dodecyl sulfate-PAGE) that has been stained with Coomassie Blue. (b) FvTox1 was detected with mouse anti-FvTox1 monoclonal antibody; (c) *E. coli* expressed Anti-FvTox1-1 was used as primary antibody; (d) *E. coli* expressed Anti-FvTox1-2 was used as primary antibody; and (e) *E. coli* expressed GmPUB1 protein (30 kDa) served as negative control for primary antibodies. CF, *F. virguliforme* culture filtrates; Bv, recombinant FvTox1 protein expressed in baculovirus infected Sf21 insect cell line; Vc, protein preparation from Sf21 insect cell line infected with baculovirus carrying empty vector. *E. coli* expressed scFv antibodies were tagged with Xpress epitope. Binding of *E. coli*-expressed Anti-FvTox1 scFv antibodies to FvTox1 was detected using anti-Xpress antibody (Invitrogen, Carlsbad, CA).
Figure 2 Specificity of binding properties of Anti-FvTox1 scFv antibodies expressed in transgenic soybean roots. (a) Diagram depicting the truncated forms of FvTox1. Region between the two arrows [based on results shown in (c) below] contains the antigenic site for the mice monoclonal Anti-FvTox1 7E8 antibody used in this study. (b) Coomassie Blue stained gel of proteins expressed in E. coli. Western blot analysis of the E. coli expressed proteins using (c) monoclonal anti-FvTox1 antibody 7E8 or proteins extracted from: (d) A. rhizogenes-induced transformed roots carrying Anti-FvTox1-1 gene with KDEL-tag fused to
its 5’end, (e) *A. rhizogenes*-induced transformed roots carrying *Anti-FvTox1-2* gene with *KDEL*-tag fused to its 5’end, (f) *A. rhizogenes*-induced transformed roots carrying *Anti-FvTox1-1* gene with *KDEL*-tag fused to its 3’end, (g) *A. rhizogenes*-induced transformed roots carrying *Anti-FvTox1-2* gene with *KDEL*-tag fused to its 3’end. 140, mature-*FvTox1*(33-172aa); 172, Pro-*FvTox1*(1-172aa); 58, 58 aa long pro-*FvTox1* protein truncated at the C-terminus; 74, 74 aa long pro-*FvTox1* protein truncated at the C-terminus; 120, 120 aa long pro-*FvTox1* protein truncated at the C terminus; *U-Box*, an *E. coli* expressed soybean *U-Box* protein; E, empty lane; CF, *F. virguliforme* culture filtrate.
Figure 3 Progenies of a stable transgenic soybean plant carrying Anti-FvTox1-1 showed reduced foliar SDS development following infection with *F. virguliforme*. (a) Non-transgenic Williams 82 showing higher levels of foliar SDS; (b) Transgenic plants showing reduced foliar SDS development; (c) A non-transgenic Williams 82 plant showing susceptible foliar SDS symptoms in young trifoliates. (d) A transgenic plant carrying Anti-FvTox1-1 antibody gene showed enhanced tolerance to the pathogen infection and produced healthy trifoliates. (e) Reduced foliar SDS symptom development in transgenic plants as compared to that in non-transgenic control. The plants were scored as follows. 0, no symptoms; 1, < 10% chlorosis; 2, 10 to 20% chlorosis; 3, 20-50% chlorosis; 4, 50-80% chlorosis and necrosis; 5, entire leaf became chlorotic or necrotic. (f) Enhanced growth rates of transgenic plants carrying Anti-FvTox1-1 over that of non-transgenic Williams 82 plants. SL, shoot length; RL, root length; PW, average fresh weight of the entire plant; RW, average fresh root weight. Error bars show the standard errors.
Figure 4 Progenies of a stable transgenic soybean line carrying Anti-FvTox1-1 showed reduced foliar SDS development. (a) Foliar SDS symptoms in non-transgenic (left) and transgenic (right) plants that were fed with cell-free Fv culture filtrates. (b) Close-up picture of a diseased leaf of a non-transgenic plant. (c) Close-up photo of a leaf of a transgenic plant fed with the cell-free Fv culture filtrate. (d) Foliar symptoms development in two non-transgenic (left) and two transgenic plants (right) 14 days following feeding with cell free Fv culture filtrates. (e) Quantification of foliar SDS symptoms. Chlorophyll content (mg/cm³) and disease score were determined for the non-transgenic and transgenic soybean plants to
determine the extents of foliar SDS development following feeding with \textit{Fv} culture filtrates. The plants were scored as described in Figure 3.

\textbf{Figure 5} Co-segregation of tolerance to FvTox1 with the integration of an \textit{Anti-FvTox1-1} transgene copy. (a) Segregation of progenies of a transformant for tolerance to FvTox1. The progeny shown with an arrow was the only susceptible plant (S1). (b) Western blot showing expression of Anti-FvTox1-1 scFv antibody (arrow). R1-R5, SDS-resistant progenies; S1, SDS-susceptible progeny; NR, non transgenic soybean root; TR, transgenic soybean root; NT; non transgenic soybean leaf; P1, a progeny that was not evaluated for SDS-resistance. (c) Southern blot showing the \textit{EcoRI} fragment (shown by an arrow) that was associated with the expression of tolerance to FvTox1.
Figure 6 Sub-cellular localization of Anti-FvTox1-1 scFv antibody. Anti-FvTox1-1 was localized to cytoplasm and the chloroplasts (shown by arrows) by anti-KDEL primary antibody and gold conjugated goat anti-mouse IgG secondary antibody in leaves. a) to c) transgenic plants; d) non-transgenic plants. CW-cell wall; V-Vacuole; Cyt-cytoplasm; Cl-chloroplast; S- starch grains; IS- intercellular space.
Supplementary Figure 1 Strategy in synthesizing the Anti-FvTox1 scFv antibody genes.

(a) Diagrammatic representation of an antibody molecule. Grey area marked by VL (single domain antibody) and VH (single domain antibody) contains the antigen binding site. C, constant regions of heavy and light chains; V, variable regions of heavy (H) and light (L) chains. The positions of universal primers used to amplify the variable regions are shown by the arrows; Fab, fragment antigen binding antibody. (b) Diagrammatic representation of the structure of an scFv molecule. Two PCR amplified variable fragments are joined by a polylinker encoding three repeat peptides of four glycine and one serine residue. (c) PCR amplification of variable heavy and light chains. 1, heavy chain, 8-1; 2, Light chain, 4-2; 3, light chain, 6-1. (d) Generation of two scFv molecules by joining heavy and light chains with a polylinker sequence. 1, Anti-FvTox1-1 (scFv 8-1/4-2); 2, Anti-FvTox1-2 (scFv 8-1/6-1).
Supplementary Figure 2 The comparison of heavy chain 8-1 with similar sequences from NCBI database. The CDR regions have been boxed. "*" represents identical residues; ":" means conserved substitutions between similar residues; "." indicates the semi-conserved substitutions between similar residues.
**Supplementary Figure 3** The comparison of light chain 4-2 with similar sequences from NCBI database. The CDR regions have been boxed. "*" represents identical residues; ":" means conserved substitutions between similar residues; "." indicates the semi-conserved substitutions between similar residues.

**Supplementary Figure 4** The comparison of light chain 6-1 with similar sequences from NCBI database. The CDR regions have been boxed. "*" represents identical residues; ":" means conserved substitutions between similar residues; "." indicates the semi-conserved substitutions between similar residues.
Supplementary Figure 5 Isolated Anti-FvTox1 variable heavy and light chain fragments carry complimentary determining regions. The complimentary determining regions (CDR) regions of cloned variable regions were detected using the Kabat database based numbering scheme. The heavy chain, 8-1 and the light chain 4-2 has 3 CDRs each; the light chain, 6-1 contains only 2 CDRs suggesting that it may be partial length. The boxed residues indicate the position of conserved cysteine residues.

Supplementary Figure 6 Expression of Anti-FvTox1 scFv antibodies in E. coli (a) Coomassie blue stained gel of proteins extracted from E. coli expressing 1, Anti-FvTox1-1 scFv antibody; 2, Anti-FvTox1-2 scFv antibody; M, Marker. (b) Western blot analysis of E. coli expressed 3, Anti-FvTox1-1 scFv antibody; 4, Anti-FvTox1-2 scFv antibody.
Supplementary Figure 7 Expression of Anti-FvTox1 scFv proteins in transformed soybean roots. (a) Generation of adventitious roots on detached 10-day old soybean cotyledons 15 days following infection with *A. rhizogenes*. (b) PCR analysis of DNA extracted from transformed roots induced by *A. rhizogenes*. Forward primers (8-1F KDEL) from *Anti-FvTox1* genes and the reverse primer (PolyATailR) from the binary vector were used in PCR (Supplementary Table 1). (c) RT-PCR analysis of transcripts extracted from transformed roots using *Anti-FvTox1*-specific forward (8-1F KDEL) and the binary vector-specific reverse primer (PolyATailR) (Supplementary Table 1). (d) RT-PCR analysis as in (c) but
with no reverse transcriptase added to the RT reaction. (e) Western blot analysis of Anti-FvTox1 antibodies expressed in transformed roots. Anti-KDEL antibody was used to conduct the western blot analyses. M, protein marker; 1, KDEL fused to the N-terminus of Anti-FvTox1-1; 2, KDEL fused to the N-terminus of Anti-FvTox1-2; 3, KDEL fused to the C-terminus of Anti-FvTox1-1; 4, KDEL fused to the C-terminus of Anti-FvTox1-2, Ev1, DNA from adventitious roots generated following infection with A. rhizogenes carrying an empty binary vector.

**Supplementary Figure 8** Binding properties of soybean root-expressed Anti-FvTox1 scFv antibodies to FvTox1 (a) Coomassie blue stained gel; (b) through (f), western blot analyses to determine the binding properties of Anti-FvTox1 antibodies to FvTox1. Protein preparations for western blot analyses were from: (b), A. rhizogenes-induced untransformed roots, (c) A. rhizogenes-induced transformed roots carrying Anti-FvTox1-1 gene with KDEL tag fused to its 5’end, (d) A. rhizogenes induced transformed roots carrying Anti-FvTox1-2 gene with KDEL tag fused to its 5’end, (e) A. rhizogenes induced transformed roots carrying Anti-FvTox1-1 gene with KDEL tag fused to its 3’end, (f) A. rhizogenes-induced transformed roots carrying Anti-FvTox1-2 gene with KDEL tag fused to its 3’end. CF, F. virguliforme
culture filtrates; Bv, Baculovirus expressed pro-FvTox1 in the SF21 insect cell line; Vc, empty vector containing baculovirus infected SF21 cell line.

Supplementary Figure 9 Enhanced growth of transgenic soybean plants expressing Anti-FvTox1-1 antibody. (a) Non-transgenic Williams 82 seedlings showing suppressed seedling growth; (b) enhanced growth of progenies of a transformant carrying the AntiFvTox1-1 antibody gene. Analyses of these plants are presented in Figure 4.
CHAPTER 4 LOCALIZATION OF FVTOX1 IN *FUSARIUM VIRGULIFORME* INFECTED SOYBEAN LEAF TISSUES

Abstract

Sudden death syndrome (SDS) caused by the fungal pathogen *Fusarium virguliforme* (*Fv*) is an emerging serious disease in soybean. The typical SDS symptoms consist of leaf chlorosis and necrosis, defoliation and shedding of flowers, pods and root rot. The fungus infects the root system and has never been isolated from the diseased tissues of the above ground soybean plants. The pathogen releases a proteinaceous toxin, FvTox1 into the soybean roots. The toxin travels presumably through the vascular bundle and causes foliar SDS symptoms initially in leaves. Not much is known about the mechanism of action of FvTox1. As a first step towards understanding the mechanism used by the toxin to cause foliar SDS, I investigated the cellular location, in which the toxin accumulates following *F. virguliforme* infection of soybean roots. The leaves of soybean plants showing foliar SDS symptoms were harvested 21 days following infection with *F. virguliforme* spores and used in conducting immunohistochemistry (ICC). FvTox1 was localized to chloroplasts of the leaves that started to show chlorosis. No cross reactivity of the monoclonal anti-FvTox1 antibody 7E8, used in this study, to any soybean proteins was detected in healthy leaves of uninfected soybean plants. The FvTox1 was localized to the chloroplasts of dying cells. This observation suggested that FvTox1 most likely disrupts the photosynthetic machinery leading to chlorosis and necrosis in foliar soybean tissues.
Introduction

Soybean is one of the major crops grown in the United States. Sudden death syndrome (SDS) is a serious disease of soybean causing yield losses of about a million tons annually which is valued to about 300 million dollars (Wrather and Koenning, 2006). A soil borne fungus *Fusarium virguliforme* has been identified as the causal agent of the disease (Roy et al., 1989; Rupe, 1989; Aoki et al., 2003). *F virguliforme* is a soil borne fungus that attacks the soybean root systems and causes chlorosis and necrosis in the foliage tissues. *Fv* is a soil borne fungus that attacks the soybean root systems and causes chlorosis and necrosis in the foliage tissues (Roy et al., 1989; Rupe, 1989). The pathogen has never been isolated from the diseased tissues of the above ground parts of the soybean plants. The fungus releases a proteinaceous toxin, *FvTox1*, into the roots of the soybean plants that moves through the vascular system to the shoots to cause the foliar SDS or leaf scorch (Brar et al., 2010).

Numerous fungi have been reported to produce proteinaceous toxins. *Pyrenopora tritici–repentis* (Ballance et al., 1989; Tomas et al., 1990; Tuori et al., 1995; Zhang et al., 1997); *Alternaria* spp. (Otani et al., 1998; Quayyum et al., 2003); *Ceratocystis fimbriata* (Pazzagli et al., 1999; Pazzagli et al., 2006); *Fusarium* spp. (Bailey, 1995; Sutherland and Pegg, 1995; Bailey et al., 2000; Brar et al., 2010) and *Stagonospora nodorum* (Lui et al., 2004; Friesen et al., 2006; Friesen et al., 2007; Friesen et al., 2008; Abeysekara et al., 2009) are some of the fungi that produce proteinaceous toxins.

The studies on *Ptr ToxA* toxin, produced by a wheat pathogen *Pyrenopora tritici–repentis*, have shown that *Ptr ToxA* has an RGD (Arginine, Glycine, and Asparagine) cell attachment motif, similar to the mammalian protein Vitronectin (Manning et al., 2004).
RGD motif helps in importing Ptr ToxA into the wheat cells. Ptr ToxA has been shown to be localized to the cytoplasmic compartments and in the chloroplast (Manning and Ciuffetti, 2005). Ptr ToxA interacts with a chloroplast localized protein named ToxABP1 (Manning et al., 2007).

Earlier studies in Bhattacharyya Lab have shown that the cell-free Fv culture filtrates, when fed to the soybean seedlings, caused degradation of the large subunit of ribulose-1,5-bisphosphate carboxylase-oxygenase in the presence of light (Ji et al., 2006). It was hypothesize that FvTox1 disrupts the photosynthetic machinery (Ji et. al., 2006). In this study I investigated the sub-cellular location of leaf cells, into which FvTox1 accumulates. Identification of the sub-cellular location will provide us a clue about the way FvTox1 does its function.

**Results**

**FvTox1 was detected in the Fusarium virguliforme infected soybean leaf tissue**

A western blot analysis was conducted on the leaf and root tissues from Fv infected soybean plants, but FvTox1 was not detected in any of the samples (Supplementary Figure 1). Immunohistochemistry of leaf tissues from *F. virguliforme*-infected and uninfected soybean plants of the cultivar Williams 82 was conducted by using DAB staining. The monoclonal anti-FvTox1 7E8 antibody detected FvTox1 in the soybean leaf tissues of the *F. virguliforme*-infected plants (Figure 1A). In the absence of anti-FvTox1 antibody, no FvTox1 was detected in the leaf tissues of the *F. virguliforme* -infected plants (Figure 1B). In the
healthy leaf tissues of water control Williams 82 plants the antibody did not show cross-reactivity to any soybean proteins.

**FvTox1 was localized to the chloroplasts**

Light microscopy was conducted on the DAB stained foliar tissues that were hybridized to the anti-FvTox1 7E8 antibody. The FvTox1 was localized to the chloroplasts. The size of the organelle, its number in individual cells and comparison of the bright field pictures with the phase contrast pictures (Figure 2B) suggested that FvTox1 accumulated in the chloroplasts. No DAB staining was observed in sub-cellular locations other than the chloroplasts.

**Accumulation of FvTox1 was associated with the dying cells**

Localization of FvTox1 by DAB staining showed that accumulation of the toxin was always occurred in chloroplasts of dying cells (Figure 3A). We failed to detect FvTox1 in intact cells that were close to dying cells containing FvTox1 (Figure 3B). This observation suggested that FvTox1 most likely causes cell death.

**Discussion**

In our study, through western blot analysis we were not able to detect the FvTox1 in the *F. virguliforme* infected soybean tissues (Supplementary Figure 1). FvTox1 was detected in chloroplasts, when we conducted immunohistochemical analyses (Figures 2-4). The samples for microscopy were taken from plants that showed severe foliar SDS symptoms. FvTox1
was detected however only in a few leaf cells (Figures 2-4) suggesting that a few molecules of FvTox1 are required to activate the process of disease development.

Earlier study has shown that in presence of light, FvTox1, degrades the large subunit of Ribulose-1, 5-Biphosphate Carboxylase-Oxygenase (Rubisco) (Ji et al., 2006). Rubisco is a major enzyme that participates in the Calvin cycle, which takes place in the stroma of chloroplast. The cell-free *F. virguliforme* culture filtrates when fed to cut soybean seedlings lead to production of reactive oxygen species (ROS) in the presence of light (Ji et al., 2006). Light is also essential for symptom production in leaf tissue by the baculovirus expressed FvTox1. Earlier studies have shown that Victorin (a toxin produced by an oats pathogen, *Cochliobolus victoriae*) can degrade Rubisco (Navarre and Wolpert, 1999). Rubisco degradation leads to disruption of chloroplast electron transport chain, leading to accumulation of ROS in the chloroplast. Earlier it was shown that chloroplasts under stress are capable of producing ROS (Mullineaux and Karpinski, 2002; Doyle et al., 2010). The ROS production in chloroplast has been shown to cause programmed cell death in tobacco (Chen and Dickman, 2004) and *Arabidopsis* (Doyle et al., 2010). After internalization of FvTox1 into the chloroplast, most likely it interacts with soybean protein(s) to disestablish the photosynthesis, and thereby to produce ROS for initiating cell death and foliar SDS. Chloroplast localization of FvTox1 supports this hypothesis.

**Material and methods**

**Soybean infection with Fusarium virguliforme**

Soybean seeds were grown in a 1:1 mixture of sand and soil containing *Fusarium virguliforme* inocula grown on sorghum seeds according to (Mueller et al., 2002). Control
plants were grown only in sand and soil mixture. The seedlings were allowed to germinate in the growth chambers under light at 25°C in for 16 h and in dark at 16°C for 8 h. The light intensity was 200 μmol photons m⁻² sec⁻¹. The foliar tissue samples were harvested 23 days after infection for microscopy.

**Tissue fixation and sectioning**

Leaf punches were taken from *F. virguliforme*-infected and healthy 23 days old soybean plants. The samples were fixed in 0.1M Cacodylate buffer containing 3% paraformaldehyde and 0.5% glutaraldehyde for 4 h. The samples were washed thrice with 0.1 M Cacodylate buffer and dehydrated with pure xylene. The samples were embedded with paraffin for two days and casted into molds. The samples were cut into 8 μm thick section using a rotary microtome and fixed onto poly-L-Lysine coated slides by using Sta-on solution (Surgipath, Richmond, IL).

**DAB staining**

A DAB kit (Dako, Glostrup, Denmark) was used for indirect detection of FvTox1 in the paraffin fixed sections. The slides were heated at 55°C for 30 min and blocked with peroxidase block for 6 min. Anti-FvTox1 monoclonal antibody was used as the primary antibody. The biotin-streptavidin linkage was done according to a standard protocol (Dako, Glostrup, Denmark). The slides were incubated in the DAB substrate for 15 min. The slides were dehydrated with pure xylene and mounted with permount.
**Light microscopy**

A Zeiss Axioplan microscope (Carl zeiss, Germany) was used to acquire images during bright field and phase contrast microscopy. For higher magnification oil emersion lens was used (Carl Zeiss, Germany).

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**References**


Figure 1. Localization of FvTox1 in *Fusarium virguliforme*-infected foliar tissues. A) Cross reaction of anti-FvTox1 7E8 antibody to FvTox1 suggested FvTox1 accumulates in chloroplasts (arrows); B) No reaction to the reagents with no primary antibody was detected in control healthy tissues. C) No cross-reactivity of anti-FvTox1 antibody was detected in control healthy tissues.
**Figure 2.** FvTox1 was localized to the chloroplasts (arrows). A) Bright-field light microscopy; B) Phase-contrast microscopy. No FvTox1 was detected in any regions other than the chloroplasts.
Figure 3. FvTox1 is associated with degrading leaf cells. A) FvTox1 (arrows) detected in a layer of cells showing disintegrating chloroplasts; B) High magnification of FvTox1 in chloroplasts of degrading cells. The intact cell within the circle showed absence of FvTox1.
Supplementary Figure 1. Western blot analysis of proteins extracted from *F. virguliforme* infected (*Fv+*) and *F. virguliforme* uninfected (*Fv−*) soybean root and leaf tissue. A) Western blot for proteins extracted from soybean leaves. DAI, days after inoculation, CF, *F. virguliforme* culture filtrates; B) Loading differences in protein amounts of samples analyzed in A. Note that the amount of proteins in cell free-CF was very small. C) Western blot of protein samples extracted from soybean roots. D) Loading differences in protein amounts of samples analyzed in C. Arrows indicate the FvTox1 detected in the cell free-CF.
CHAPTER 5 GENERAL CONCLUSIONS

In this study I have isolated a proteinaceous toxin, FvTox1 from *Fusarium virguliforme* and showed that it can induce foliar SDS symptoms. In Chapter 2, the isolation and characterization of the toxin, FvTox1, has been discussed. The putative toxin FvTox1 was purified by gel filtration followed by PAGE of concentrated cell-free *F. virguliforme* culture filtrates. The purified FvTox1 was used to raise anti-FvTox1 monoclonal antibodies in mice. An expression cDNA library constructed from *F. virguliforme* mycellial RNAs was screened using the anti-FvTox1 antibodies and the FvTox1 cDNA was isolated. FvTox1 was expressed through baculovirus infection of an insect cell line, Sf21. The expressed FvTox1 protein was infiltrated into discs prepared from unifoliate leaves of three weeks old soybean seedlings. The baculovirus expressed FvTox1 lead to loss of chlorophyll and necrosis-like symptoms, when it was infiltrated into the soybean leaf discs. Leaf discs of the SDS-susceptible soybean cultivars, but not those of SDS-resistant soybean cultivars were sensitive to the expressed FvTox1 protein.

Chapter 2 also describes the characterization of the FvTox1 gene. A *F. virguliforme* genomic library was screened using the FvTox1 cDNA molecule. Several clones were isolated from the genomic library. Restriction enzyme analysis of the clones showed that they all belonged to a single class. FvTox1 is a small gene of 627 bp containing three exons. The two small introns are 61 and 50 bp long. The Southern blot analysis and analysis of the *F. virguliforme* genome sequence revealed that FvTox1 is a single copy gene.

In Chapter 3, the PCR amplification and assembly of two Anti-FvTox1 scFv antibodies (Anti-FvTox1-1 and Anti-FvTox1-2) genes has been discussed. The antibody genes were cloned from RNAs isolated from the hybridoma cell lines expressing the anti-FvTox1
7E8 monoclonal antibody. *Escherichia coli* expressed antibodies encoded by these genes were shown to bind FvTox1. A DNA sequence encoding the KDEL sequence was fused to 5’- or 3’-ends of both Anti-FvTox1 antibody genes and four fusion genes were created and cloned into the binary vector, pISUAgron5. *Agrobacterium rhizogenes* carrying the binary vector constructs were used to transform soybean cotyledons and generate transgenic hairy roots. The proteins from transgenic roots were tested for their specificities of binding to FvTox1. Both scFv antibodies carrying the KDEL tag at their C-termini and Anti-FvTox1-2 carrying the tag at its N-terminus were able to bind FvTox1. However, due to reasons unknown, Anti-FvTox1-1 carrying the KDEL tag at its N-terminus failed to bind FvTox1. The anti-FvTox1-1 antibody gene carrying KDEL tag at its C-terminus was used to generate stable transgenic soybean plants. The progenies of one of the transformant were analyzed for possible tolerance to FvTox1. The progenies of the transformant showed enhanced tolerance to FvTox1 and resistance to *F. virguliforme* as compared to the non-transgenic control soybean plants. Following root inoculation with the pathogen, the average disease score of the progenies of the transformant was significantly lower than that of the wild type Williams 82 control plants. The root length, stem length and fresh weight were significantly higher in transgenic plants as compared to that in non-transgenic control plants. Following feeding of cut soybean seedlings with *F. virguliforme* culture filtrates, higher chlorophyll contents and lower foliar SDS disease scores were observed for the transgenic plants as compared to the non-transgenic Williams 82 control plants.

In Chapter 4, localization of FvTox1 in infected soybean leaf tissues has been presented. Immunohistochemistry of *Fv*-infected leaf tissues was conducted by DAB staining with the aid of the monoclonal anti-FvTox1 7E8 antibody. FvTox1 was localized to the
chloroplasts in *F. virguliforme*-infected soybean leaf tissues. FvTox1 accumulated in the chloroplast of dying cells. No FvTox1 was detected in intact cells, indicating that FvTox1 most likely initiates cell death. Since an earlier study had shown that Rubisco large subunit was degraded in leaf tissues of soybean cut seedlings fed with cell-free *F. virguliforme* culture filtrates containing FvTox1, chloroplast was speculated to be the target of FvTox1 (Ji et al. 2006). Most likely degradation of the photosynthetic machinery starts just after internalization of FvTox1 into the chloroplast and in presence of light. Disruption of photosynthetic machinery leads to accumulation of reactive oxygen species, which initiate programmed cell death process, observed in foliar SDS.

In my research I have shown that FvTox1 is the toxin involved in SDS-like symptom development in soybean leaf discs and Anti-FvTox1-1 scFv antibody confers enhanced tolerance in transgenic soybean plants against FvTox1. Therefore, I conclude that FvTox1 is the major or key pathogenicity factor for foliar SDS development. Outcomes of my research provides both basic information and tools for investigation of the signal pathways involved in foliar SDS development and creating SDS resistant soybean germplasms. It is still unknown how the FvTox1 is internalized into the chloroplasts? What soybean proteins are involved in interaction with FvTox1? What signaling pathways participate in SDS foliar symptom development?

In an earlier study it was shown that cell-free *F. virguliforme* culture filtrates are capable of degrading Rubisco large subunit in presence of light suggesting that host factor(s) are involved in foliar SDS development. Thus the investigation of mechanism of SDS disease development may be initiated by using a yeast two hybrid approach to isolate soybean protein(s) that interact with FvTox1. Once the interacting soybean proteins are isolated, their
characterization might lead to elucidation of the mechanism of foliar SDS development in soybean.