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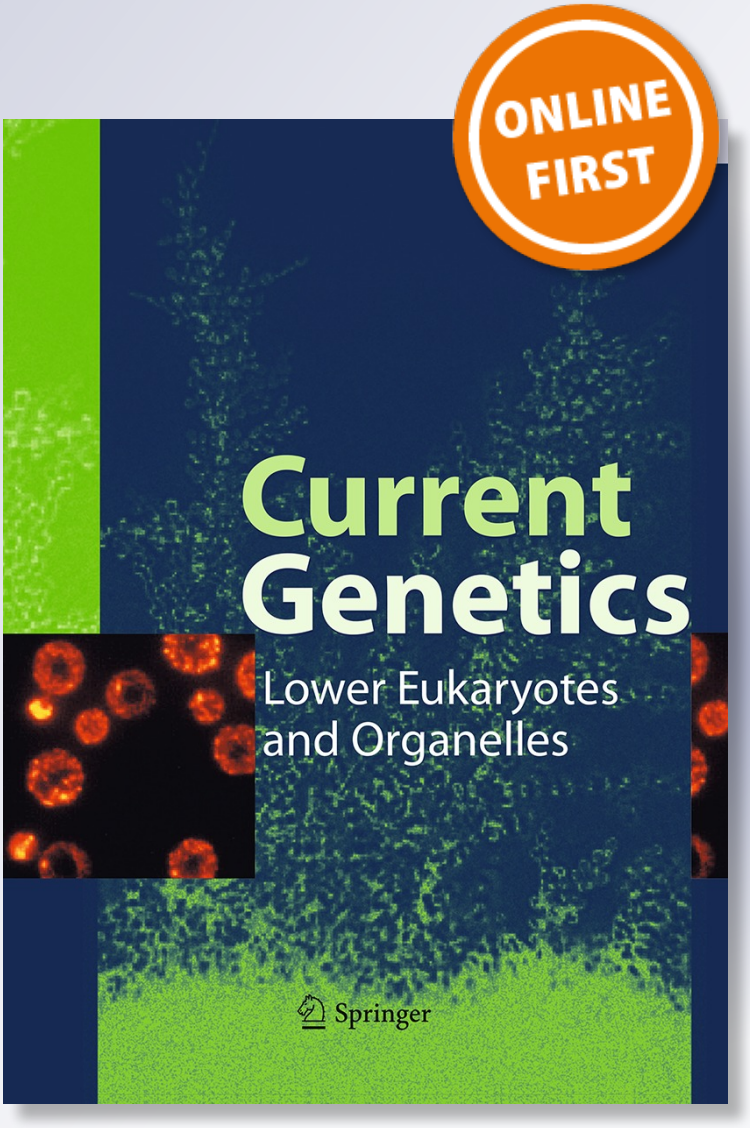

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RESEARCH ARTICLE

Investigation of the *Fusarium virguliforme fvtox1* mutants revealed that the FvTox1 toxin is involved in foliar sudden death syndrome development in soybean

Ramesh N. Pudake · Sivakumar Swaminathan · Binod B. Sahu · Leonor F. Leandro · Madan K. Bhattacharyya

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Abstract The soil borne fungus, *Fusarium virguliforme*, causes sudden death syndrome (SDS) in soybean, which is a serious foliar and root rot disease. The pathogen has never been isolated from the diseased foliar tissues; phytotoxins produced by the pathogen are believed to cause foliar SDS symptoms. One of these toxins, a 13.5-kDa acidic protein named FvTox1, has been hypothesized to interfere with photosynthesis in infected soybean plants and cause foliar SDS. The objective of this study is to determine if FvTox1 is involved in foliar SDS development. We created and studied five independent knockout *fvtox1* mutants to study the function of FvTox1. We conducted *Agrobacterium tumefaciens*-mediated transformation to accomplish homologous recombination of *FvTox1* with a hygromycin B resistance gene, *hph*, to generate the *fvtox1* mutants. Approximately 40 hygromycin-resistant transformants were obtained from 10^6 conidial spores of the *F. virguliforme* Mont-1 isolate when the spores were co-cultivated with the *A. tumefaciens* EHA105 but not with LBA4044 strain carrying a recombinant binary plasmid, in which the *hph* gene encoding hygromycin resistance was flanked by 5′- and 3′-end *FvTox1* sequences. We observed homologous recombination-mediated integration of *hph* into the *FvTox1* locus among five independent *fvtox1* mutants. In stem-cutting assays using cut soybean seedlings fed with cell-free *F. virguliforme* culture filtrates, the knockout *fvtox1* mutants caused chlorophyll losses and foliar SDS symptoms, which were over twofold less than those caused by the virulent *F. virguliforme* Mont-1 isolate. Similarly, in root inoculation assays, more than a twofold reduction in foliar SDS development and chlorophyll losses was observed among the seedlings infected with the *fvtox1* mutants as compared to the seedlings infected with the wild-type Mont-1 isolate. These results suggest that FvTox1 is a major virulence factor involved in foliar SDS development in soybean. It is expected that interference of the function of this toxin in transgenic soybean plants will lead to generation of SDS-resistant soybean cultivars.

Keywords *Fusarium virguliforme* · *FvTox1* · *Agrobacterium tumefaciens* · *ATMT*-mediated homologous recombination · pRF-HU2 · Knockout mutants

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Introduction

In the United States, soybean is the second most important row crop after corn. It is estimated that soybean suffers yield reduction valued over 2.3 billion dollars annually from various diseases including sudden death syndrome (SDS) (Wrather and Koenning 2006). SDS was first detected in Arkansas in 1971 (Hartman et al. 1995), and is

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now prevalent in all soybean growing states (Brar et al. 2011). The disease is caused by *Fusarium virguliforme*, a haploid ascomycete (Aoki et al. 2005). The pathogen causes both root necrosis and foliar leaf chlorosis and necrosis leading to defoliation and pod drop. Although the foliar disease symptoms are responsible for severe yield losses, the pathogen has never been isolated from the above ground diseased tissues. It has been hypothesized that the pathogen secretes one or more toxins to infected roots that are presumably responsible for the development of foliar SDS symptoms. However, molecular evidence is lacking in support of the role of any toxins for foliar SDS development in soybean.

It has been considered that one or more toxins are secreted into the culture media; and when the cell-free culture filtrates containing these toxins are fed to cut soybean seedlings, SDS-like foliar symptoms are developed (Li et al. 1999). Recently, a 13.5-kDa protein FvTox1 was isolated from the cell-free *F. virguliforme* culture filtrates that produce SDS-like foliar symptoms in soybean leaf discs (Brar et al. 2011). Furthermore, transgenic soybean plants expressing a single chain variable fragment (scFv) anti-FvTox1 antibody created against the toxin were tolerant to FvTox1 (Brar and Bhattacharyya 2012). In this study, we created and analyzed *fvtox1* mutants to establish the possible role of FvTox1 in foliar SDS development.

To create knockout *fvtox1* mutants, we applied an *Agrobacterium tumefaciens*-mediated homologous recombination approach that has been successfully applied in other *Fusarium spp*. It has been shown that homologous recombination is very effective in knocking out genes in filamentous fungi such as *Fusarium graminearum*, *Botrytis cinerea*, *Nectria haematococca*, and *Aspergillus fumigatus* (Krappmann et al. 2006; Garmaroodi and Taga 2007; Have et al. 1998; Frandsen 2011). Replacement of target genes with selectable marker genes through homologous recombination results in complete loss of target genes in haploid organisms. Such mutants are ideal for identifying functions of target genes. Transformation procedures including protoplast transformation and *A. tumefaciens*-mediated transformation (ATMT) have greatly facilitated the development of homologous recombination methodologies in fungi. ATMT has been widely used in diverse group of fungi (de Groot et al. 1998; Dobinson et al. 2004; Michielse et al. 2005). ATMT produces transformants that carry only a few T-DNA insertions as opposed to the protoplast transformation and particle bombardment techniques that result in a large number of transgene insertions (Frandsen et al. 2011; Michielse et al. 2009; Malz et al. 2005; Abba et al. 2009).

Recently, a rapid vector construction system for generating recombinant molecules for homologous recombination from four different DNA fragments was developed and successfully applied in *F. graminearum* (Frandsen et al. 2008, 2011). In this system, the promoter and terminator regions of a target gene are cloned on either side of the hygromycin resistance gene, *hph*, in a binary vector plasmid. *In vivo* double crossing-over in the promoter and terminator regions of a target gene in the genome with those in the T-DNA molecule resulted in replacement of the target gene with the *hph* gene. In the present study, we successfully applied such a system (Frandsen et al. 2008) for creating knockout *fvtox1* mutants. We studied randomly selected five independent knockout *fvtox1* mutants and observed that FvTox1 is a major virulence factor essential for foliar SDS development in soybean.

Materials and methods

Fungal isolate and growth conditions

A highly virulent isolate, *F. virguliforme* Mont-1 was maintained on Bilay solid medium (Brar et al. 2011). Following 3 weeks of growth, single mycelial plugs were transferred onto plates containing 1/3 PDA solid medium (Brar et al. 2011), and incubated in dark at room temperature (RT) for 3 weeks for conidia production. The conidial spores were collected by adding sterile water to the sporulating plates and used for co-cultivation with *A. tumefaciens* carrying the binary vector for transformation on solid IMAS medium (Frandsen et al. 2008) containing acetosyringone (0.2 mM).

Construction of a knockout vector for *F. virguliforme* transformation

The strategy to knockout a target gene is shown in Supplemental figure 1a. The two homologous recombination segments (HRS) ($\sim$1.3 kb each) representing promoter and termination regions of the *FvTox1* gene were selected based on the sequence information available at the *F. virguliforme* genome database (http://fvgbrowse.agron.iastate.edu), and were PCR amplified. Primers used for first HRS (T-O1 and T-O2), second HRS (T-O3 and T-O4) are listed in Table 1. The HRS were cloned in flanking sites of the *hph* cassette of the pRF-HU2 binary vector (Frandsen et al. 2008) using USER enzyme mix (New England Biolab, Inc., Ipswich, MA, USA) in *Escherichia coli*. The resultant plasmid was confirmed for proper orientation of cloned inserts in the vector by PCR conducted using HRS- and vector-specific primers and then by sequencing the PCR products.

Agrobacterium tumefaciens-mediated transformation

The pRF-HU2::Δ*FvTox1* binary plasmid containing two *FvTox1*-specific HRS was transformed into *A. tumefaciens*

Table 1 Oligos used in this study

Name	Sequence	Product size (bp)
T-O1	GGTCTTAAUGGACGCCGATACCAACTCAAACTGGACGTC	1,382
T-O2	GGCATTAAUGCCGAGATTCAACGGCAGTCCATCACCTTC	
T-O3	GGACTTAAUCGGGCACAGGGATACACCAGAGGAGGAAC	1,313
T-O4	GGGTTTAAUCCGCGCTGTTCTCTTCCATCGTAGCCATTAC	
Hyg588U	AGCTGCGCCGATGGTTTCTACAA	588
Hyg588L	GCGCGTCTGCTGCTCCATACAA	
T-I-F	GGCACCACGCCTGAGGAGTACGATC	483
T-I-R	CTACTGTGGGTTGCGCACACAG	
g4748F	CCAACGTCACCACTGAAGTCAAGTC	290
g4748R	GGTCGATCTTCTCCTGGATCTC	
LBF	CGAATTCACTGGCCGTCGTTTTAC	250
LBR	CGCTTAGACAACTTAATAACACATTG	
RF-1	AAATTTTGTGCTCACCGCCTGGAC	NA
RF-2	TCTCCTTGCATGCACCATTCCTTG	
T-U	CTTCCCAAGGTTGAAAGGACGG	NA
T-D	TAGTCTTCCTCTGCGTCGTGCC	

NA not applicable

strains EHA105 and LBA4404 by electroporation, and transformants were analyzed by conducting restriction analysis. The ATMT of *F. virguliforme* was based on a published protocol (Frandsen et al. 2008). Briefly, *A. tumefaciens* LBA4404 and EHA105 containing pRF-HU2::Δ*FvTox1* plasmid were grown overnight in LB medium at 28 °C (50 µg/ml kanamycin and 25 µg/ml rifampicin). The next day, 10 ml of IMAS medium (25 mg/ml of kanamycin) was inoculated with 100 µl of the *A. tumefaciens* culture. This *A. tumefaciens* cell suspension with an OD_{600} of 0.5–0.7 was mixed with *F. virguliforme* Mont-1 conidial suspensions (2×10^6/ml) in liquid IMAS medium in equal proportions [1:1(v/v)]. Aliquots of 200 µl of the mixture were spread on black filter paper circles (Grade 551; Whatman Inc., Piscataway, NJ, USA), which were overlaid on IMAS plates and incubated for 3 days in the dark at RT until mycelial growth was observed on the filter paper. Transformants were selected on defined *Fusarium* medium (DFM) (Frandsen et al. 2008) supplemented with 150 µg/ml of hygromycin B (Sigma, St. Louis, MO, USA) and 300 µg/ml cefoxitin (Fisher Scientific, Pittsburgh, PA, USA). Colonies from both the primary and secondary selection plates were aligned and the selected independent colonies were transferred onto the new plates for single spore isolation for further investigation.

PCR analyses of *F. virguliforme* transformants

For polymerase chain reaction (PCR) analysis, DNA was isolated from mycelia grown from single spore derived colonies in liquid MSM medium (Brar et al. 2011) using cetyl trimethyl ammonium bromide (CTAB) buffer (Sambrook et al. 2001). The integration of the *hph* gene was confirmed by PCR using the *hph*-gene specific primers (Hyg588U and Hyg588L, Table 1). Loss of the *FvTox1* gene in *fvtox1* mutants was determined by PCR using primers specific to coding regions of *FvTox1* (T-I-F and T-I-R, Table 1). A total of 38 *F. virguliforme* genes (http://fvgbrowse.agron.iastate.edu/) were amplified by PCR (Table 1; Supplemental table 1).

Southern blot analysis of the *fvtox1* mutants

Five micrograms of genomic DNA of each *fvtox1* mutant and Mont-1 were digested with either *Xba*I or *Xba*I and *Eco*RI (New England Biolab, Inc., Ipswich, MA, USA) at 37 °C overnight. The pRF-HU2 and pRF-HU2::Δ*FvTox1* plasmids were digested with *Pac*I and *Xba*I at 37 °C overnight. The restricted DNA fragments were separated in 0.8 % (w/v) agarose gels and transferred onto charged nylon membranes by alkaline transfer (Sambrook et al. 2001). Probes specific to *FvTox1*, *hph*, *FvTox1*-promoter, *FvTox1*-terminator and left T-DNA border were amplified by PCR (Supplemental figure 1b and Table 1). Labeling reactions were carried out with the Prime-a-Gene labeling system (Promega, Madison, WI, USA) using ^{32}P-α dATP (Perkin Elmer, Waltham, MA, USA). Southern hybridization and washing were conducted at high stringency and blots were exposed to X-ray film (Sambrook et al. 2001).

Sodium dodecyl sulfate polyacrylamide gel electrophoresis and western blot analysis

Cell-free *F. virguliforme* culture filtrates were prepared as described earlier (Brar et al. 2011). Total protein estimation

of cell-free *F. virguliforme* culture filtrates was done using the Bradford protein assay (Bradford 1976). Fifteen microgram of total proteins prepared from culture filtrates were separated on a 12 % (w/v) sodium dodecyl sulfate polyacrylamide gel at 80 V for 2 h and then either stained with Coomassie blue G-250 or electro-blotted onto PROTRAN nitrocellulose membrane (Whatman Inc., Piscataway, NJ, USA) at 30 V for 15 h at 4 °C. After blocking, the membrane was probed with the anti-FvTox1 7E8 monoclonal antibody (Brar et al. 2011). Hybridization was detected using goat anti-mouse antibody conjugated to alkaline phosphatase (Bio-Rad, Hercules, CA, USA) and the NBT/BCIP substrate (Bio-Rad, Hercules, CA, USA) as per manufactures' instructions.

Stem-cutting assay of *fvtox1* mutants

The stem-cutting assay with cell-free *F. virguliforme* culture filtrates was carried out as previously described (Ji et al. 2006; Li et al. 1999; Brar et al. 2011). Soybean 'Williams 82' seedlings were grown in a growth chamber for 3 weeks under previously described conditions (Brar and Bhattacharyya 2012). We observed roughly 75 µg proteins in 1 ml of culture filtrate. Therefore, FvTox1 cell-free culture filtrates of individual isolates containing 75 µg total proteins were diluted in sterile water to 25 ml in 50 ml tube and used for stem-cutting assays of single soybean cut seedlings (Brar and Bhattacharyya 2012). The seedlings fed with only diluted MSM medium were treated as controls. These seedlings were then incubated under the previously described growth conditions (Brar and Bhattacharyya 2012). In general, SDS-like symptoms started to appear in the seedlings after 5–6 days of feeding *Fv* culture filtrates. The SDS-like symptoms were scored at 7, 9, 11, 13 and 15 days following the feeding with culture filtrates as follows: 0, no symptom; 1, leaves showing general slight yellowing and/or chlorotic flecks or blotches; 2, leaves with obvious, interveinal chlorosis; 3, leaves with necrosis along a portion (>2 cm) of its margin; 4, necrosis along the entire margin of leaves and the leaves are curled with irregular shapes; 5, interveinal necrosis and most (∼>50 %) of leaf area is necrotic and/or defoliation. Average scores from scores of individual plants were considered to calculate the average disease index.

After 15 days of treatment with culture filtrates, three leaf disks (1.8 cm^2) were excised from second, third and fourth trifoliate leaves (counting from the apex of each soybean seedling) and placed individually in 1.5 ml microcentrifuge tubes and frozen at −80 °C. The following day, 1 ml of 80 % acetone was added to each tube and the tubes were incubated at RT in the dark for 5 days to extract the chlorophyll. Absorbance (OD) of the acetone solution containing chlorophyll was measured at 645 and 663 nm and the amount of chlorophyll was calculated as described earlier (Arnon 1949). Ten replications were used for each isolate and experiment was repeated three times.

Root inoculation using *F. virguliforme* grown in sorghum meal

Germinating seeds of Williams 82 were inoculated with the *fvtox1* mutants and wild-type Mont-1 grown on sorghum meals (Hartman et al. 1997; de Farias Neto et al. 2008; Lightfoot et al. 2007). Five weeks before planting soybean seeds, the fungal culture was grown in 200 g of sterile sorghum seeds in 1 quart Mason jars aseptically. Ten mycelial plugs of Mont-1 and *fvtox-1* mutants from 1/3 strength PDA plate were used to infect sorghum grains and grown for 4 weeks, after which inoculated grains were ground in a blender and used for the assay. Before the assay, inoculum load of the pathogen in sorghum meal was estimated by extracting genomic DNA from the ground fungus-infested sorghum and by performing semi-quantitative PCR with primers specific to 38 *F. virguliforme* genes (Supplemental figure 2, Supplemental table 1). Based on the PCR results, equal amount of inocula from individual *fvtox1* mutants and Mont-1 was mixed with sterile soil:sand (1:2) at ratio of 1:10 (v/v) to prepare the inocula. For the root inoculation assay, clean styrofoam cups (8 oz.) were filled with 150 ml of sterile soil:sand mix and then 30 ml of the inocula in sorghum meals. Three seeds of Williams 82 were placed on the top of the inoculum and covered with another 30 ml of soil:sand mix. Fifteen days after sowing and growing the seedlings in a growth chamber, foliar SDS symptom development was recorded in a 3-day interval and until 27 days following planting. Scoring SDS symptom and chlorophyll estimation (27 days after planting) was carried out as described for the stem-cutting assay. This experiment was conducted three times with ten replications for each isolate in each experiment. The seedlings grown in sterile sorghum meal without the pathogen were used as a negative control.

Results

A. tumefaciens strain-specific transformation of *F. virguliforme* Mont-1 isolate

The pRF-HU2 vector containing the selectable marker *hph* gene for hygromycin resistance and HRS for homologous recombination (Supplemental figure 1a) was transformed into two *A. tumefaciens* strains, EHA105 and LBA4404. Transformation of *F. virguliforme* Mont-1 with *A. tumefaciens* EHA105 resulted in 44 ± 1.5 transformants per 10^6 conidial spores. There were no transformants when the

A. tumefaciens LBA4404 strain was used for transformation.

Molecular analysis of the *F. virguliforme* transformants

Five putative transformants generated from independent transformation events were selected for further analyses. Single conidium derived colonies were selected from individual transformants. Initially, PCR was conducted using primers specific to *hph* and *FvTox1* genes (Table 1). The *hph*-specific PCR fragments were amplified from all five putative mutants suggesting that the selected colonies were most likely transformants. The *FvTox1*-specific primers amplified *FvTox1*-specific DNA fragments only from the wild-type Mont-1 isolate. A random *F. virguliforme* gene, g4748, was amplified form all five putative mutants (Fig. 1a). Subsequently, we conducted PCR with the primers specific to 37 additional *F. virguliforme* genes to establish that all five independent putative mutants were generated from *F. virguliforme* (Supplemental figure 2, Supplemental table 1). All PCR results suggested that the selected five mutants were generated from the *F. virguliforme* Mont-1 isolate.

To characterize the mutants, primers were designed from upstream and downstream regions of the HRS as shown in Supplemental figure 1b (Primers T-U and T-D). These primers along with *hph*-specific primers were used to amplify the recombinant molecules (Fig. 1b), which can only be possible from homologous recombination-based replacement of the target *FvTox1* gene with the *hph* gene. Sequencing of these PCR fragments confirmed that the recombinant genomic locus carrying *hph* was created from all five mutants due to homologous recombination, and that the promoter and terminator regions of FvTox1 were intact.

Southern blot analysis was conducted to support the PCR results. To our surprise, all five *fvtox1* mutants carried only a single *hph*-specific band (Fig. 2a). No second *hph*-hybridizing DNA fragment was observed among any of the five *fvtox1* mutants. Therefore, it is most likely that the *hph* gene was integrated only into the *FvTox1* locus. We did not observe any hybridization of the *FvTox1* gene (open reading frame)-specific probe to the genomic DNA of *fvtox1* mutants (Fig. 2b). Thus, based on PCR and Southern analyses, we concluded that *FvTox1* was replaced by *hph* among the selected five transformants. To support our conclusion, we also used probes specific to HRS and showed that as expected (Supplemental figure 1b), two *Xba*I fragments, instead of one as in Mont-1 isolate, were observed in all five mutants (Fig. 2c). Southern blot analysis of genomic DNA, double digested with *Xba*I and *Eco*RI, also revealed similar results (Fig. 2d). These results showed that *FvTox1* gene was replaced by the *hph* gene in

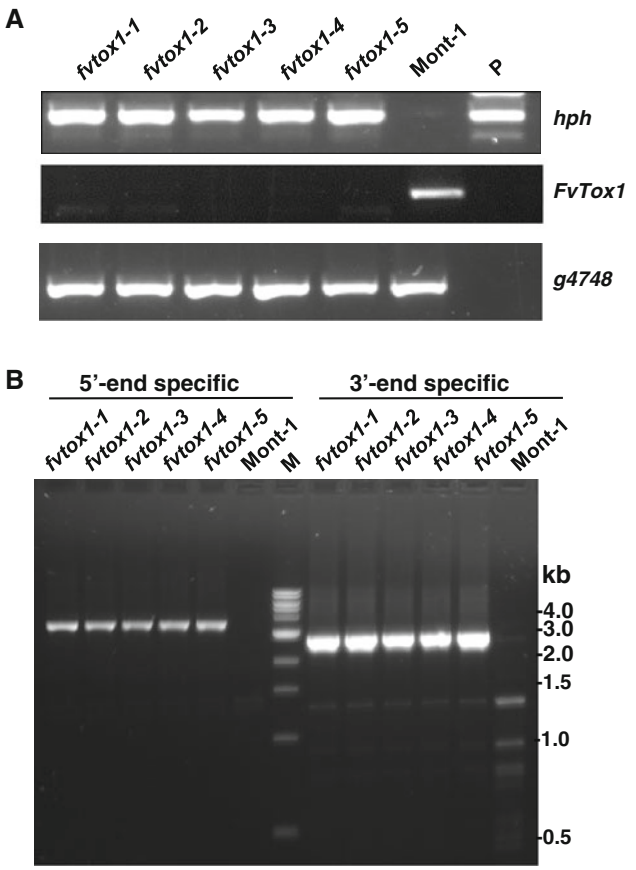

Fig. 1 PCR analysis of *fvtox1* mutants. **a** *Fvg4748* gene was amplified using g4748F and g4748R primers (290 bp); *hph*, hygromycin resistance B gene, was amplified by Hyg588U and Hyg588L primers (588 bp); *FvTox1* was amplified by T-I-F and T-I-R primers (483 bp). Primer sequences are presented in Table 1. *fvtox1-1* to *fvtox1-5*, five *fvtox1* mutants; Mont-1, the wild-type *F. virguliforme* Mont-1 isolate; and P, pRF-HU2::*FvTox1* plasmid. **b** PCR amplification of recombinant molecules generated through homologous recombination between HRS in pRF-HU2::*FvTox1* and endogenous-HRS sequences. Strategy for PCR amplification of *FvTox1* HRS is shown in Supplemental figure 1. In the *fvtox1* mutants, the promoter region was amplified using T-U and RF-2 and the terminator region using RF-1 and T-D primers (Table 1)

the five independent transformants. We termed the transformants as *fvtox1* mutants, *fvtox1-1* through *fvtox1-5*.

Absence of non-homologous end joining among the *F. virguliforme* transformants

The *hph* gene was integrated into the *FvTox1* locus through homologous recombination. To determine if there was any non-homologous end joining integration of the T-DNA molecules among the five *fvtox1* mutants, we conducted PCR using primers specific to left and right borders in conjunction with the *hph*-specific primers. No PCR amplification was observed when T-DNA border-specific primers were used along with *hph*-specific primers. These

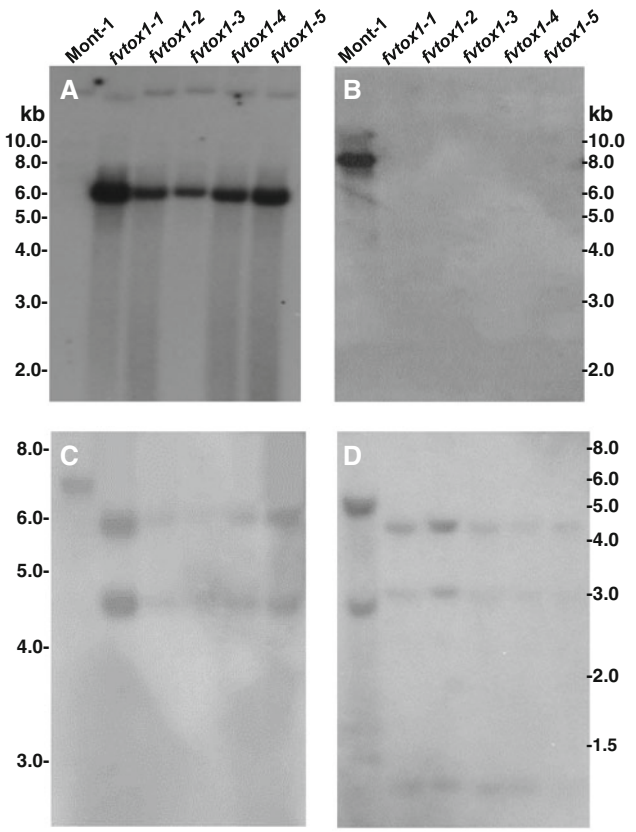

Fig. 2 Southern blot analysis of *fvtox1* mutants. **a** and **b** Genomic DNA samples from Mont-1 as well as the five *fvtox1* mutants were digested with *Xba*I and separated in a 0.8 % agarose gel. The DNA blots were probed with *hph* (588 bp) and *FvTox1* probes (483 bp), respectively (Table 1). The expected *Xba*I DNA fragments hybridized to *hph* and *FvTox1* probes were 6.6 and 7.6 kb, respectively (Supplemental figure 1b). Blots carrying *Xba*I (**c**) and *Xba*I and *Eco*RI (**d**)-digested genomic DNA were hybridized to a mixture of the promoter and terminator-specific probes as shown in Fig. 1. The expected *Xba*I and *Eco*RI DNA fragments of Mont-1 are 4.3 and 3.3 kb, respectively

results suggest the absence of T-DNA integration among the *fvtox1* mutants (data not shown). To confirm the PCR results, we conducted Southern blot analysis of the *fvtox1* mutants for the left T-DNA border-specific probe and no hybridization signal was observed among the mutants (Supplemental figure 3). These results and integration of a single *hph* molecule into the *FvTox1* locus (Fig. 2a) suggest that T-DNA is only involved in double homologous recombination for replacing the *FvTox1* gene with the selectable marker *hph* gene

Lack of FvTox1 expression among the *fvtox1* mutants

To support the results of Southern blot analysis, we determined the extent of FvTox1 expression among the *fvtox1* mutants by conducting western blot analysis with mouse monoclonal anti-FvTox1 7E8 antibody (Brar et al. 2011). As expected, we did not detect any expression of FvTox1 protein among the *fvtox1* mutants (Fig. 3).

Reduced foliar SDS caused by *fvtox1* mutants

To determine the role of FvTox1 in foliar SDS development, we studied all five independent *fvtox1* mutants by conducting the stem-cutting and root inoculation assays (de Farias Neto et al. 2008; Li et al. 1999). In stem-cutting assays, the severity of SDS-like symptom development caused by cell-free *fvtox1* culture filtrates was over twofold lower than that by the cell-free culture filtrates of the wild-type Mont-1 isolate (Fig. 4b; Supplemental figure 4). Furthermore, reduced severity in foliar SDS symptoms among the diseased leaves of cut soybean seedlings fed with cell-free *fvtox1* culture filtrates was associated with over twofold less losses in chlorophyll contents as compared to that of the diseased leaves of seedlings fed with cell-free *F. virguliforme* Mont-1 culture filtrates (Fig. 4c).

Inoculation of soybean roots with *F. virguliforme* sorghum inocula revealed that the soybean seedlings infected with the five *fvtox1* mutants remain healthy (Fig. 5a) and the extent of foliar SDS symptom development was over twofold less than the symptoms developed following

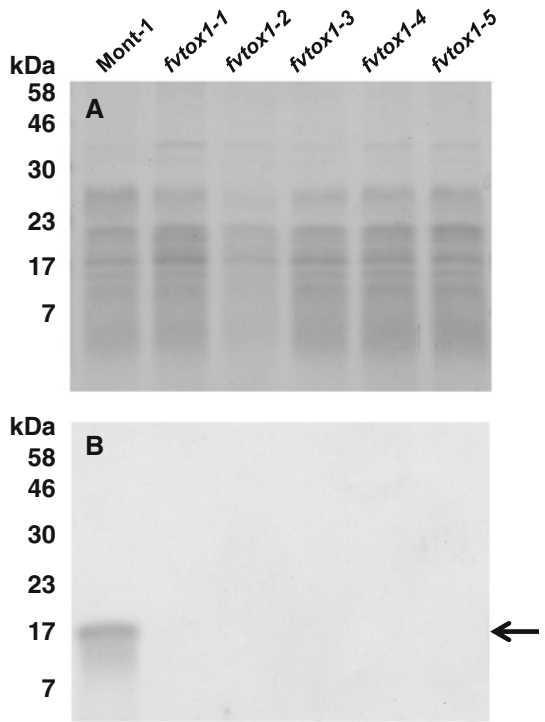

Fig. 3 Western blot analysis of the *fvtox1* mutants. **a** 15 µg proteins of culture filtrates prepared from each isolates were separated by SDS-PAGE (12 % acrylamide) and stained with Coomassie Blue; **b** gel was blotted and hybridized to the monoclonal anti-FvTox1 (7E8) antibody. The ~17 kDa FvTox1 protein (shown by an *arrow*) was detected only in Mont-1 but not among the five *fvtox1* mutants

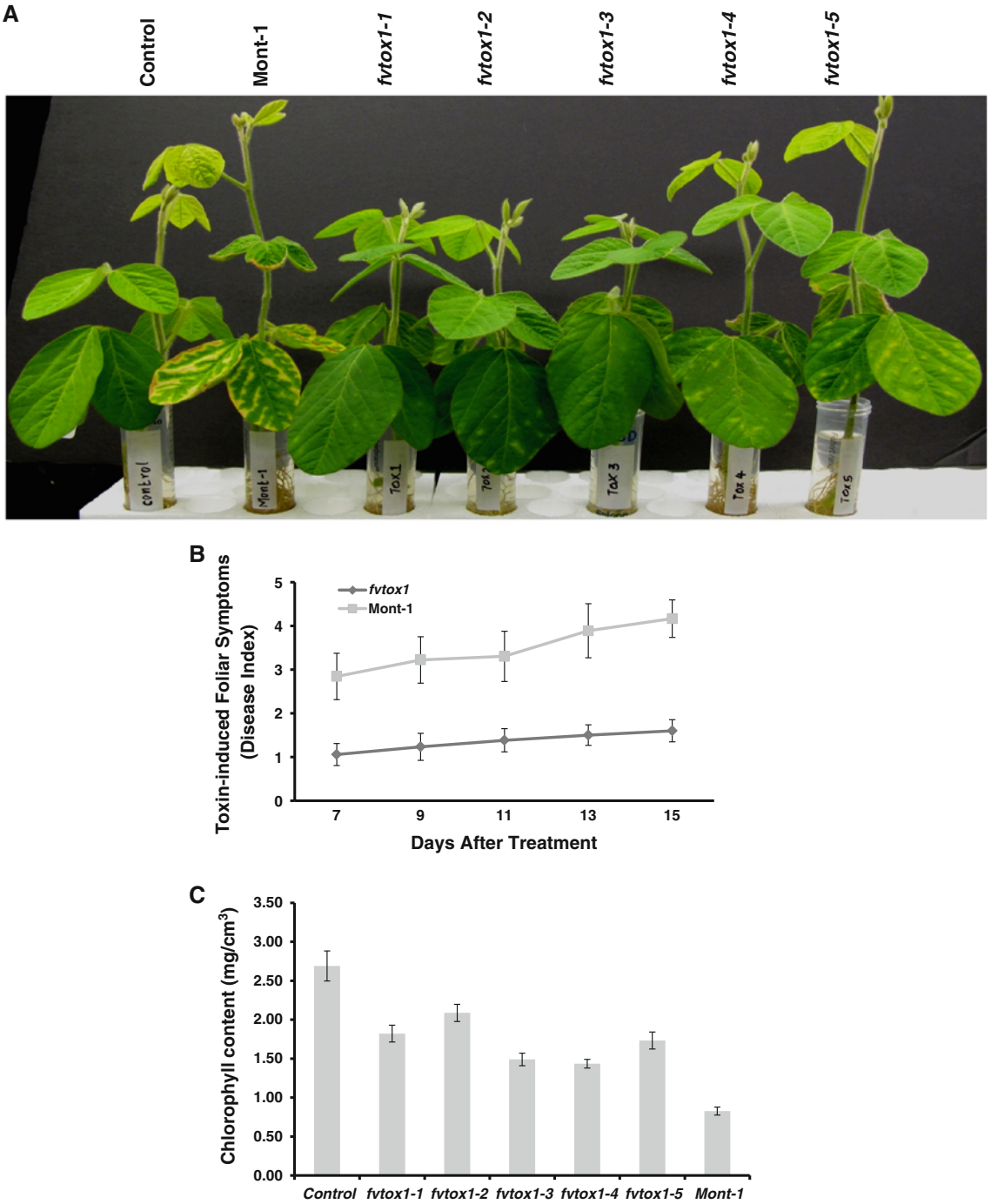

Fig. 4 Foliar SDS symptom development in cut soybean seedlings fed with cell-free *F. virguliforme* culture filtrates. **a** Severe foliar SDS symptoms developed following feeding of cut soybean seedlings with cell-free Mont-1 culture filtrates and seedlings fed with the cell-free *fvtox1* culture filtrates were almost symptom free. One representative infected seedling is presented for each isolate. **b** Culture filtrates of *fvtox1* mutants produced over twofold less foliar SDS symptoms compared to the wild-type Mont-1 isolate. Mean disease severity caused by culture filtrates of all five mutants is presented. **c** More than twofold more chlorophyll content was recorded among the soybean seedlings fed with cell-free *fvtox1* culture filtrates compared to the seedlings fed with cell-free Mont-1 culture filtrates. The control seedlings were fed with only water

inoculation with the Mont-1 isolate (Fig. 5b; Supplemental figure 5). The reduced foliar SDS symptom development in the *fvtox1* mutant was also associated with an over twofold reduction in chlorophyll contents as compared to the seedlings infected with the *F. virguliforme* Mont-1 isolate (Fig. 5c).

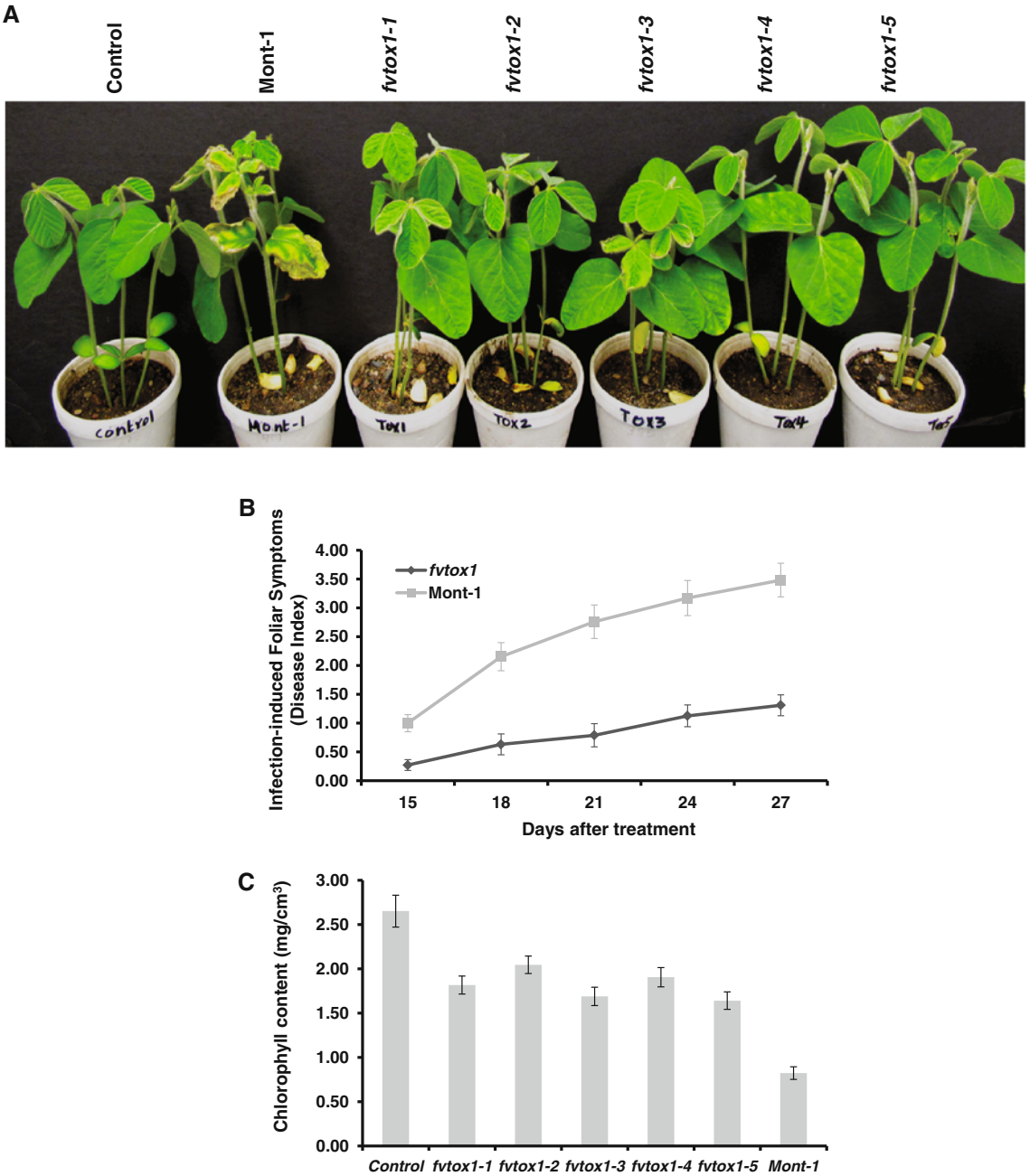

Fig. 5 FvTox1 is required for foliar SDS development. **a** Severe foliar SDS symptoms developed in soybean seedlings grown in Mont-1 infested soil. Seedlings were almost symptom free when grown in soil infested with *fvtox1* mutants. **b** Soybean seedlings infected with the *fvtox1* mutants produced over twofold lesser foliar SDS disease symptoms as compared to that by the Mont-1 isolate. Mean disease severity caused by all five mutants is presented. **c** More than twofold more chlorophyll contents were recorded among the seedlings inoculated with the *fvtox1* mutant fungus compared to the seedlings inoculated with the Mont-1 isolate. The control seedlings were grown in sterile sorghum meal without the pathogen

Discussion

A. tumefaciens-mediated replacement of *FvTox1* with *hph* in *F. virguliforme* through homologous recombination

The filamentous fungus *F. virguliforme* is a serious pathogen and causes SDS in soybean. Recently, the whole genome of this fungal pathogen was sequenced (http://fvgbrowse.agron.iastate.edu). Here, we report the targeted replacement of the *F. virguliforme FvTox1* gene with *hph* through homologous recombination via ATMT. We also observed that ATMT-mediated transformation of the *F. virguliforme* was specific to the *A. tumefaciens* strain.

Earlier, a polyethylene glycol-mediated protoplast transformation procedure was reported for this pathogen (Mansouri et al. 2009). The major disadvantage of protoplast transformation is the integration of multiple inserts in the genome. ATMT has been widely applied in transforming *Fusarium* spp. (Frandsen 2011; Malz et al. 2005; Frandsen et al. 2008; Mullins et al. 2001). Data from PCR, Southern blot and western blot analyses (Figs. 1, 2, 3) clearly established that in all five independent transformants, *FvTox1* was replaced with the *hph* gene.

Lack of or poor non-homologous end joining of the T-DNA molecules in *F. virguliforme*

A lower frequency of non-homologous end joining for *Fusarium* spp. as compared to that in other fungal species, such as *Neurospora crassa* and *A. nidulus*, has already been reported (Frandsen 2011).

In *Fusarium oxysporum* f. sp. *lycopersici*, when the ATMT-mediated gene replacement was conducted for the *AVR1* gene, the frequency of homologous recombination was observed to be extremely low; only a single knockout mutant was identified from screening of 200 transformants (Houterman et al. 2008). In *F. graminearum*, the frequencies of double crossover for the *aurZ*, *aurT* and *aurS* loci were 11, 78 and 74 %, respectively, when ATMT was applied for homologous recombination (Frandsen et al. 2011). On the contrary, 100 % homologous recombination with no evidence of non-homologous end joining was observed in *Fusarium fujikuroi* (Fernandez-Martin et al. 2000).

The frequency of homologous recombination has been reported to be highly dependent on the mode of transformation (Davidson et al. 2000). For example, the frequency of homologous recombination is lower among transformants obtained by electroporation as compared to that by ATMT (Adachi et al. 2002). Efficiency of gene knock-out by homologous recombination in fungi can also be improved by decreasing the expression of genes involved in non-homologous end joining. For example, using this approach, the frequency of homologous recombination was increased up to 100 % in *A. fumigates* and *N. crassa* (Ninomiya et al. 2004; Krappmann et al. 2006). In *Magnaporthe grisea*, deletion of the *mus52*/*KU80* gene for non-homologous end joining increased the frequency of homologous recombination up to 80 % (Villalba et al. 2008).

Here, we have presented molecular evidence of 100 % homologous recombination for the *FvTox1* locus. Southern blot analyses of knockout 21 additional mutants for five additional *F. virguliforme* genes also revealed 100 % homologous recombination in this fungus (J. Baumbach, C.D. Brooke, R. N. Pudake and M.K. Bhattacharyya, unpublished). Predominant homologous recombination, lack of evidence for non-homologous end joining repair and possible *A. tumefaciens* strain-specific transformation may be some of the reasons why successful ATMT transformation for the random integration of T-DNA molecules into the *F. virguliforme* genome has not yet been reported.

The FvTox1 is an important virulence factor for foliar SDS development in soybean

Many plant pathogens exert their destructive effects by secreting low molecular weight toxic compounds. For example, fungal secondary metabolites alone can cause some or all symptoms of a disease. In recent years, a large number of novel fungal phytotoxins and their modes of action have been identified (Aboukhaddour et al. 2012; Choquer et al. 2007, Liao and Chung 2008; Heiser et al. 2004; Dayan et al. 2008). FvTox1 has been considered to be a major virulence factor of *F. virguliforme* (Brar and Bhattacharyya 2012; Brar et al. 2011). It was shown that FvTox1 expressed in an insect cell line causes necrosis only in the presence of light and that SDS-susceptible soybean cultivars are highly sensitive to FvTox1 (Brar et al. 2011). Expression of a scFv anti-FvTox1 antibody resulted in enhanced foliar SDS resistance among the transgenic soybean plants (Brar and Bhattacharyya 2012).

In the present study, in all five independent *fvtox1* knockout mutants, the *FvTox1* ORF was replaced with *hph*. We determined the FvTox1 expression levels among the *fvtox1* mutants using the mouse monoclonal anti-FvTox1 7E8 antibody (Fig. 3b; Brar et al. 2011). As expected, no FvTox1 was detected among the *fvtox1* mutants (Fig. 3b). Both stem-cutting and root inoculation assays of five independent *fvtox1* mutants established that FvTox1 is a major virulence factor involved in foliar SDS development in soybean (de Farias Neto et al. 2008; Li et al. 1999) (Figs. 4, 5; Supplemental figures 4, 5). We, however, observed that *fvtox1* mutants do produce foliar SDS symptoms to a much lesser extent as compared to the wild-type Mont-1 isolate (Fig. 5) suggesting that additional factors are most likely involved in the foliar SDS development in soybean. Further studies will be required to identify these factors for a better understanding of foliar SDS development in soybean.

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