INSECT RESISTANCE TO SUGAR BEET PESTS MEDIATED BY A *BETA VULGARIS* PROTEINASE INHIBITOR TRANSGENE

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Abstract:

We transformed sugar beet (*Beta vulgaris*) hairy roots and *Nicotiana benthamiana* plants with a *Beta vulgaris* root gene (*BvSTI*) that codes for a serine proteinase inhibitor. *BvSTI* is a root gene cloned from the F1016 breeding line that has moderate levels of resistance to the sugar beet root maggot (*Tetanops myopaeformis*), a major pest of sugar beet. Root maggot damage deforms roots and predisposes them to other pests and pathogens that reduce yield and quality. Transformants had high levels of *BvSTI* or GUS gene expression driven by the constitutive 35S or the BvSTI gene specific promoter, respectively. Polyacrylamide gel electrophoresis zymograms revealed clear zones that corresponded to proteinase inhibitor (PI) activities at ~24, 26 and 28 kDa in the sugar beet *BvSTI* transformants and 26 and 28 kDa in the *N. benthamiana* plants. Several insect pests of sugar beet and tobacco were bioassayed for resistance on the *BvSTI*-transgenic plant materials. *Spodoptera frugiperda* (fall armyworm), *Spodoptera exigua* (beet armyworm) and *Manduca sexta* (tobacco hornworm) larvae that were fed tobacco leaves or sugar beet roots that express the *BvSTI* gene exhibited higher mortality rates or were delayed in growth and development relative to control larvae. Since serine proteases comprise the major digestive enzymes in root maggot midguts, our findings suggest that the *BvSTI* gene is likely involved in root maggot resistance mechanisms in the F1016 genotype.

Introduction:

Assimilation of dietary proteins is critical to insect survival; therefore, inhibition of digestive proteolytic enzymes presents itself as a desirable target for development of effective strategies to control insect pests. Digestive proteases in insect midguts have been grouped into several mechanistic classes based on the amino acid residue or metal ion involved in peptide bond catalysis. Serine proteases are primarily associated with the Lepidoptera species, cysteine proteases with Coleoptera and some Homoptera, and both serine and aspartyl protease activities have been reported in midguts of Diptera (Matsumoto et al, 1995; Pendola and Greenberg, 1975). Plant proteinase inhibitors (PI) are a group of naturally occurring enzymes that are characterized by varied specificity toward proteases (Abe et al., 1994; Brzin et al., 1998; Christeller et al., 1998; Jongsma & Bolter, 1997). PIs often accumulate in tissues in response to wounding or herbivory and have been shown to have an integral role in natural plant defense mechanisms. A number of genes encoding plant PIs have been cloned and, when expressed in transgenic plants, shown to enhance insect and, in some cases, nematode tolerance (Abdeen et al. 2005; Boulter et al., 1990; Charity et al. 2005; Cowgill et al., 2002; Delledonne et al., 2001;

Duan et al., 2001; Graham et al., 1997; Maheswaran et al. 2007; Mehlo et al. 2005; Ninkovic et al., 2007; Samac and Smigocki, 2003).

Some of the most important pests of sugar beet include the sugar beet root maggot (SBRM, *Tetanops myopaeformis* Roder), fall armyworm (FAW, *Spodoptera frugiperda*), beet armyworm (BAW, *Spodoptera exigua*) and sugar beet root aphid (SBRA, *Pemphigus populivenae)*. Current control measures for controlling these insect populations rely predominately on pesticides. In order to develop alternative approaches that are environmentally sound, we are investigating the use of PI genes in sugar beet to specifically target the digestive enzymes of these pests. The digestive proteases in SBRM, FAW, BAW and SBRA have been documented to fall predominantly into the serine class of proteases (Srinivasan et al., 2006; Wilhite et al., 2000). In our previous studies, we identified a sugar beet root gene that encodes a trypsin (serine) proteinase inhibitor (*BvSTI*) (Puthoff and Smigocki 2007). *BvSTI* was cloned from the F1016 genotype released as a breeding line with moderate levels of SBRM resistance (Campbell et al. 2000; Smigocki et al., 2008). We report on *BvSTI* gene transfer, expression and effect on insect resistance in sugar beet hairy roots and *N. benthamiana* transgenic plants.

Materials and Methods:

Plant material

Sugar beet breeding lines susceptible to sugar beet root maggot (SBRM; *Tetanops myopaeformis* Roder), F1010, and moderately resistant, F1016, were used in this study (Campbell, 1990, Campbell et al., 2000). Seeds were grown in the growth chamber at 25°C during the day and 18 to 20°C at night with a day length of 16 h. Seedlings were transferred to the greenhouse and maintained at 20 to 30°C during the day and 18 to 25°C at night with an approximate day length of 14 to 16 h. *N. benthamiana* plants were grown from seed in the greenhouse.

BvSTI gene construct and plant transformation

Gene specific primers were used to clone the full length coding sequence of *BvSTI* (Smigocki et al., 2008). *BvSTI* was fused to the 35S promoter in the binary plasmid pCAMBIA 1301 plant transformation vector (pBvSTI) that carries the HPTII selectable marker gene for selection of hygromycin (Hg) resistant, transformed cells (CAMBIA, Canberra, Australia). *Agrobacterium rhizogenes*-mediated gene transfer was utilized to introduce the reconstructed *BvSTI* gene into sugar beet to generate transformed hairy root cultures (Kifle et al., 1999; Smigocki et al., 2008). Regenerated hairy roots were propagated *in vitro* on 1/2 B5 medium containing 5 mg Hg /l.

N. benthamiana leaf disks were transformed with *A. tumefaciens* strain EHA105 carrying the pBvSTI gene construct as previously described (Smigocki et al., 2007; Smigocki et al., 2008). Regenerated plants were transferred to the greenhouse and fertilized twice a month with Peters 20:20:20. T2 progeny plants homozygous for Hg resistance were selected from the independently derived transgenic plants and used in insect bioassay experiments.

RT-PCR analysis

Total RNA was isolated using RNeasy Plant Mini Kit (Qiagen). Titanium One-Step RT-PCR Kit (Clontech Laboratories) was used to amplify the BvSTI transgene transcripts using 100 ng of total RNA under the following conditions: 50^{o}C for 1h, 94^{o}C for 2 min 40 sec, 35 cycles of 94^{o}C for 20 sec, 58^{o}C for 40 sec, 72^{o}C for 1 min 30 sec, followed by 72^{o}C for 5 min.

Histochemical analysis of GUS expression

GUS activity was assayed by incubating plant tissues in an X-Gluc (5-bromo-4 chloro-3-indolyl-b-*d*-glucuronic acid) solution in reaction buffer containing 50 mM NaH_2PO_4 (pH 7), 0.01% Tween 20, 10 mM Na_2EDTA at 37^oC overnight (Jefferson et al., 1987). After destaining in 70% ethyl alcohol, tissues were examined for *uidA* expression under a dissecting microscope.

Proteinase inhibitor activity assays

Native proteins were extracted in ice cold 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 10 mM EDTA, 10% sucrose, 10 mM ascorbic acid, 1 mM PMSF, 2 mM DTT (Chan and De Lumex, 1982; Wang et al., 2003). The tissues were ground in liquid nitrogen and cold extraction buffer was added in proportion of 10 ml per 1 g of tissue. After centrifugation at 10,000 rpm for 10 min, the supernatant (crude extract) was concentrated to about 1 ml using Amicon Ultra 15 (3K) concentrator (Millipore, USA) by centrifugation at 4^oC. The concentrated extract was desalted in 8.5 ml of 62.5 mM TRIS, pH 6.8 two times and centrifuged until the retentate volume was less than 200 µl.

For analysis of trypsin proteinase activity, native proteins (5 µg) were separated on 12% native polyacrylamide gels. After electrophoresis, gels were incubated with gentle shaking in 25% v/v 2-propanol, 10 mM Tris-HCl pH 7.4 for 30 min to remove SDS and then in 10 mM Tris-HCl, pH 8.0 for another 30 min to re-nature the proteins. Gels were soaked with 40 µg/ml bovine trypsin in 50 mM Tris-HCl, pH 8.0, 50 mM $CaCl_2$ for 40 min and transferred to a freshly prepared substrate-dye solution consisting of 2.5 mg/ml N-acetyl-DL-phenylalanine β-naphthyl ester (Sigma) resuspended in dimethylformamide and 0.5 mg/ml tetrazotized O-dianisidine (Sigma) resuspended in 50 mM Tris-HCl (pH 8.0) with 50 mM $CaCl_2$, for 30 min at room temperature. Acetic acid (10% v/v) was added to stop the reaction. Clear zones corresponding to proteins with trypsin inhibitory activity were recorded.

Insect assays

Third instar tobacco hornworm larvae were used to infest *N. benthamiana* plants in the greenhouse. Each plant was placed in a cage with one larva. Larval weights, sizes, pupation and pupa emergence were recorded. Experiments were done in reps of 3 to 5.

Newly emerged fall and beet armyworm larvae were reared on artificial diet for 10 days. After 10 days, one larva was placed in a petri dish that contained either 1 leaf collected from greenhouse grown *N. benthamiana* plants or sugar beet hairy roots. All plant tissues were maintained on water moistened filter paper during the experiment. Larval weights were recorded daily and fresh plant material was added at regular intervals. Experiments were done in reps of 5 to 15.

Results and Discussion:

***BvSTI* gene expression**

The *BvSTI* gene was over-expressed in a number of independently derived *N. benthamiana* plants and sugar beet hairy root lines (Smigocki et al., 2008). *N. benthamiana* plants transformed with the *BvSTI* gene exhibited phenotypes that were indistinguishable from the normal, untransformed plants (Smigocki et al., 2008). Seedlings transformed with the *BvSTI* gene promoter fused to the GUS gene exhibited high levels of root specific expression as compared to the controls (Figure 1A-C). Similarly, a number of independently transformed

sugar beet hairy root lines carrying the *BvSTI* gene were regenerated from both the SBRM susceptible F1010 and moderately resistant F1016 petioles. Hairy root cultures displayed a varied pattern of root growth, which was relatively slow, moderate or rapid (Smigocki et al., 2008). Hairy root lines transformed with the GUS gene driven by the *BvSTI* gene promoter exhibited varied levels of GUS gene expression when compared to the 35S driven constitutive expression (Figure 1D).

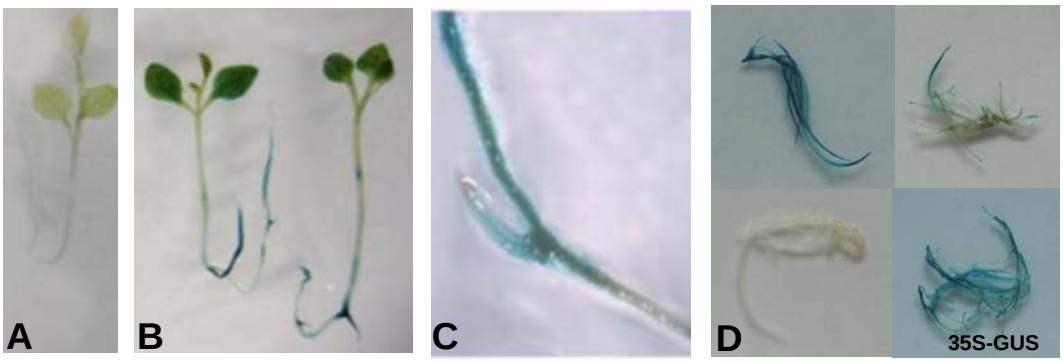

Figure 1. *N. benthamiana* seedlings transformed with the *BvSTI* gene promoter fused to the GUS gene (B and C) as compared to normal, untransformed plants (A). F1010 and F1016 hairy roots transformed with the *BvSTI* promoter-GUS gene compared to hairy roots transformed with the 35S-GUS gene construct (lower right) (D).

RT-PCR analysis of the clonal lines was used to verify gene transfer and *BvSTI* transcript levels. Transcripts were detected in all *BvSTI* transformants that were screened (Figure 2). The F1010 hairy root cultures had elevated transcript levels in the transformants but a low level of *BvSTI* transcript was also detected in the F1010 control (Figure 2A) suggesting that it may be the regulation of *BvSTI* expression that may contribute to SBRM resistance in F1016. The *N. benthamiana BvSTI* transformed plants showed a high level of transcription of the introduced sugar beet gene that was not detected in the untransformed control plants (Figure 2B).

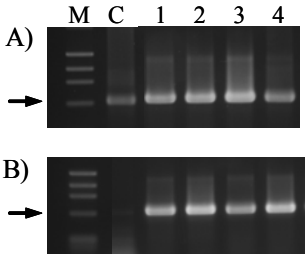

Figure 2. RT-PCR analysis of *BvSTI* gene expression in transformed (A) F1010 sugar beet hairy roots and (B) *N. benthamiana* plants. M, Lambda HindIII molecular weight markers; C, untransformed control; arrow, 0.6 kb.

BvSTI activity assays

To determine the level of proteinase inhibitor activity in the *BvSTI* transformed tissues, native protein extracts were prepared and analyzed on PAGE zymograms. In-gel trypsin inhibitory activity analysis revealed new proteinase inhibitor activity in all of the analyzed

transformants. Two independently derived F1010 sugar beet hairy root lines had activity at ~24, 26 and 28 kDa but only the 28 kDa one was not observed in the untransformed control (Figure 3A). The *N. benthamiana* plants had two of the three activities observed in the sugar beet roots, i.e. 26 and 28 kDa, but both were lacking in the untransformed control plants (Figure 3B). Presence of the recombinant protein in the transformed tissues was confirmed by Western blot analysis using polyclonal antibodies prepared from a mixture of the two most antigenic peptides of BvSTI (data not shown).

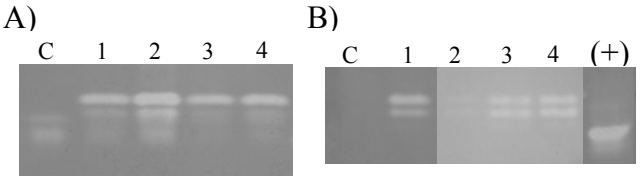

Figure 3. Trypsin inhibitor activity detected in *BvSTI* transformed (A) F1010 sugar beet hairy roots; lane 1-2, transformant 202 and lane 3-4, 207. Total protein: 5 µg in lane 1 and 3 and 10 µg in lane 2 and 4. (B) *N. benthamiana BvSTI* transformants 1-4 (5 µg total protein per lane). C, untransformed controls (5 µg); +, soybean Kunitz trypsin inhibitor, 20 kDa, 1.5 µg.

Insect Assays

N. benthamiana plants transformed with the *BvSTI* gene were used to rear tobacco hornworm (THW) larvae. A substantial reduction in the overall sizes of the larvae, pupae and adults was observed as compared to the controls (Figure 4). The same plants were also used to assay their resistance to fall armyworm. The fall armyworm is a pest that feeds on sugar beet foliage and is well-known to utilize serine protease enzymes to digest consumed plant material (Srinivasan et al., 2006). Mean larval weights of fall armyworm larvae that were fed *BvSTI* leaves were significantly lower than those of the controls after 8 days of feeding on the transformed plant materials (Table 1).

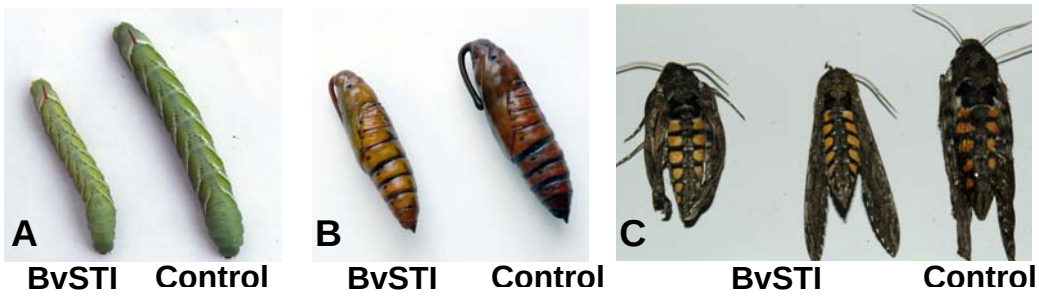

Figure 4. Tobacco hornworm larvae (A), pupae (B) and adults (C) after feeding on *N. benthamiana* plants transformed with the *BvSTI* gene (BvSTI) or on normal, untransformed (control) plants.

Table 1. Mean larval weights of fall armyworm (FAW) larvae feeding on *N. benthamiana* plants transformed with the *BvSTI* gene.

Transformant number	**Mean FAW larval weight at 8 days (mg ± SE)**
4a	76 ± 16
5a	105 ± 13
6b	74 ± 10
13	84 ± 9
1-2a	156 ± 36
control	258 ± 42

Sugar beet hairy roots similarly transformed with the *BvSTI* gene and showing high levels of proteinase inhibitor activity were bioassayed to determine the effect of the *BvSTI* gene on fall and beet armyworm feeding. A substantial reduction in mean larval weights of the beet armyworm larvae was observed at 5 days (Table 2). Interestingly, larval mortality rates ranged

Table 2. Mean larval weights of beet armyworm (BAW) and fall armyworm (FAW) larvae feeding on sugar beet hairy roots transformed with the *BvSTI* gene.

Transformed line	**Mean larval weight at 5 days – mg (percent mortality)**	
F1010	**BAW**	**FAW**
214	88 (0)	169
216	41 (100)	140
F1016		
251	76 (33)	72
259	63 (67)	103
control	105 (0)	172

from 33-100% for larvae feeding on the *BvSTI* transformed tissues while no larval deaths were observed on the controls. Results from the fall armyworm experiments indicated that larval weights were not substantially reduced after 5 days of feeding on the *BvSTI* transformed tissues (Table 2). The F1016 transformants 251 and 259 did, however, point to a reduction in larval weights as compared to the control.

Analysis of the *BvSTI* transformed tissues for resistance to one of the most important pests of sugar beet in the U.S., the sugar beet root maggot (SBRM), has not been completed.

Since SBRM cannot be reared in the laboratory, eggs and larvae are generally only available from infested fields during the summer months at which time they can be collected and utilized for insect bioassays (Smigocki et al. 2006). To date, the results from the FAW, BAW and THW bioassays suggest that the serine proteinase inhibitor encoded by the sugar beet *BvSTI* gene contributes to plant resistance mechanism(s). In addition, the results also suggest that since this gene was cloned from the roots of an SBRM resistant F1016 genotype, it likely may be involved in SBRM tolerance.

The magnitude of biotechnological advances being made in improving and developing sugar beet varieties will concomitantly intensify its cultivation and the need for effective and environmentally compatible disease and pest control strategies. Therefore, studies aimed at the characterization of genes that are involved in resistance mechanisms are of utmost importance in order to develop alternate control measures that are not based upon environmentally persistent synthetic chemistries.

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