Sugar Beet Proteinase Inhibitor (*BvSTI*) Gene Promoter is Regulated by Insects and Wounding in Transgenic *Nicotiana*

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ABSTRACT

A regulatory sequence from a serine proteinase inhibitor gene (BvSTIpro) that is induced by the sugar beet root maggot was fused to the β-glucuronidase (*GUS*) reporter gene to characterize its expression patterns in transgenic *Nicotiana benthamiana* plants. In 2- and 6-week old BvSTIpro-*GUS* transformed plants, GUS expression was observed throughout leaves and roots that was comparable to GUS staining detected in plants transformed with the positive control 35S-GUS gene construct. At 10-14 weeks, *GUS* expression in the leaves and roots was reduced as compared to the younger plants and the 35S-GUS control but was up-regulated by abiotic and biotic stress. At 6 to 24 hours after mechanical wounding, *GUS* expression increased throughout the wounded leaves and roots of 14-week old plants to constitutive levels observed with the 35S-GUS gene. Fall armyworm (*Spodoptera frugiperda)* feeding on BvSTIpro-*GUS* leaves and roots similarly induced *GUS* gene activity like those observed with the 35S-GUS control. We demonstrate that the *BvSTI* promoter induced gene expression in response to abiotic and biotic stresses. Fusion of the *BvSTI* promoter to resistance genes should provide expression at sites of pest and pathogen invasion to augment the capacity of the plant to defend itself.

Additional Keywords: sugar beet, *Beta vulgaris*, wound-inducible, promoter, proteinase inhibitor, *Nicotiana benthamiana*

Promoters that are up-regulated at a time when and where they are most needed are considered most desirable for devising effective genetic engineering strategies (Potenza et al. 2004). For plant genome modification, use of promoters derived from plant genes has often been reported to reduce or eliminate post-transcriptional gene silencing associated with promoters derived from non-plant origin (Matzke and Matzke 1995; Cramer et al., 1999). Fusion of resistance genes to powerful promoters, among them the cauliflower mosaic virus 35S (CaMV35S), maize ubiquitin or rice actin promoter, is well documented to deliver high levels of constitutive expression of transgenes in plant tissues. The continuous synthesis and relatively high accumulation of transgene products from constitutive promoters can have negative consequences, such as abnormal plant growth and metabolism (Rai et al., 2009), negative impact on nontargeted and beneficial insects (Babendreier et al., 2008) and buildup of resistance in a targeted insect population (Bakhsh et al., 2011; Tabashnik et al., 2003). For example, the constitutive expression of the widely used *Bacillus thuringiensis* (Bt) *cry* toxin increased resistance in the targeted pest to the over-produced, recombinant cry proteins (Bakhsh et al., 2011; Tabashnik et al., 2003). Tight control in a tissue specific and temporal manner via plant promoters derived from resistance genes would be expected to provide more desirable beneficial transgene expression *in planta*.

Proteinase inhibitor (PI) genes are among the many well-characterized inducible plant promoters that are derived from genes that have a role in plant defense. PI genes code for proteins that inhibit insect digestive enzymes of the midgut. PIs reduce the rate of growth and can alter insect development; therefore, they are considered highly desirable candidates that are suitable for use in crop improvement programs (Dunse et al., 2010; Maheswaran et al., 2007; Abdeen et al., 2005). Many of the studies that documented the over-expression of recombinant PIs in transgenic plants reported significant reduction or inhibition of larval growth and development (Smigocki et al., 2013; Schlüter et al., 2010; Ninkovi et al., 2007; Samac and Smigocki 2003; Telang et al., 2003). Unlike the over-production of PIs in genetically engineered plants, endogenous production of PIs has been shown to be developmentally regulated in a temporal and transient fashion, and to be up-regulated by abiotic and biotic stresses (Logemann et al., 1989; Bryant et al., 1976). The reported patterns of PI gene expression suggest that PI gene promoters may prove useful for regulating and inducing the expression of other beneficial transgenes in plants. Specifically, fusion of their promoters to resistance genes should prove effective for targeting expression to sites that are under pests and pathogen attack. Among the characterized inducible PI promoters, the potato wound-inducible (*wun1*), proteinase inhibitor II *(pin2)* and poplar *win3.12T* gene promoters were shown to direct high wound- and pathogen-inducible expression in transgenic plants (Xu et al., 1993; Keil et al., 1990; Yevtushenko et al., 2004).

In a study of sugar beet root defense responses, a sugar beet serine PI (*BvSTI*) gene was identified (Puthoff and Smigocki 2007). The importance of the BvSTI PI in insect pest defense mechanisms was demonstrated in transgenic tobacco plants that over-expressed the *BvSTI* gene from the CaMV 35S promoter (Smigocki et al., 2013). In the present study, we report on the cloning of the *BvSTI* gene regulatory sequence. We demonstrate *BvSTI* gene driven expression of the ß-glucuronidase *GUS* (*uidA*) reporter gene in *N. benthamiana* and show that it is regulated by abiotic (mechanical wounding) and biotic (insect feeding) stress stimuli. The utility of the *BvSTI* promoter for directing wound induced leaf and root expression of beneficial resistance transgenes is discussed.

MATERIAL AND METHODS

BvSTI regulatory sequence

A PCR-based strategy was employed to clone the *BvSTI* gene regulatory sequence (GenomeWalker™ Universal Kit, BD Biosciences, Palo Alto, CA). Genomic DNA was isolated from F1016 sugar beet germplasm (Campbell et al., 2000) and digested with the restriction enzymes *Dra*I, *Eco*RV, *Pvu*II or *Stu*I. GenomeWalker Adaptor sequences (provided in the kit) were ligated to each batch of digested DNA and used as template for PCR with *BvSTI* gene specific primers: 5’-CCATTTCTCAGTGCATCGCCGTCTGTGTCT-3’ and 5’-AGGAGGAGCACAGTGGTGGTTGATTTCAG-3’ in the primary and nested secondary PCR, respectively (Smigocki et al. 2013). PCR conditions were 5 cycles: 94 °C (25 s), 72 °C (3 min); 20 cycles: 94 °C (25 s), 67 °C (3 min) and 1 cycle: 67 °C (7 min). The amplified DNA fragment was sequenced and specific primers were designed for PCR amplification of the 794 bp regulatory promoter sequence upstream of the ATG start codon. Following primers were used: Forward 5’AAGCTTACTATGAAAGAAAGGAAGTAATAA3’ and Reverse 5’CCATGGTGTTTTTGTTTGGTGTG3’ with unique restriction enzyme sites *Hind*III and *Nco*I, respectively, engineered at the 5’ ends of the primers to facilitate the subcloning of the *BvSTI* promoter into a plant transformation vector. Following sequence verification, the *BvSTI* promoter fragment was fused upstream of the *GUS* (*uidA*) reporter gene with a NOS terminator in pCAMBIA1301 (BvSTIpro-*GUS*; Fig. 1A; CAMBIA, Canberra, Australia; Smigocki et al., 2013). pCAMBIA1301 carries the *hpt*II selectable marker gene coding for hygromycin phosphotransferase for selection of hygromycin (Hyg) resistant transformed plant cells (Fig. 1A).

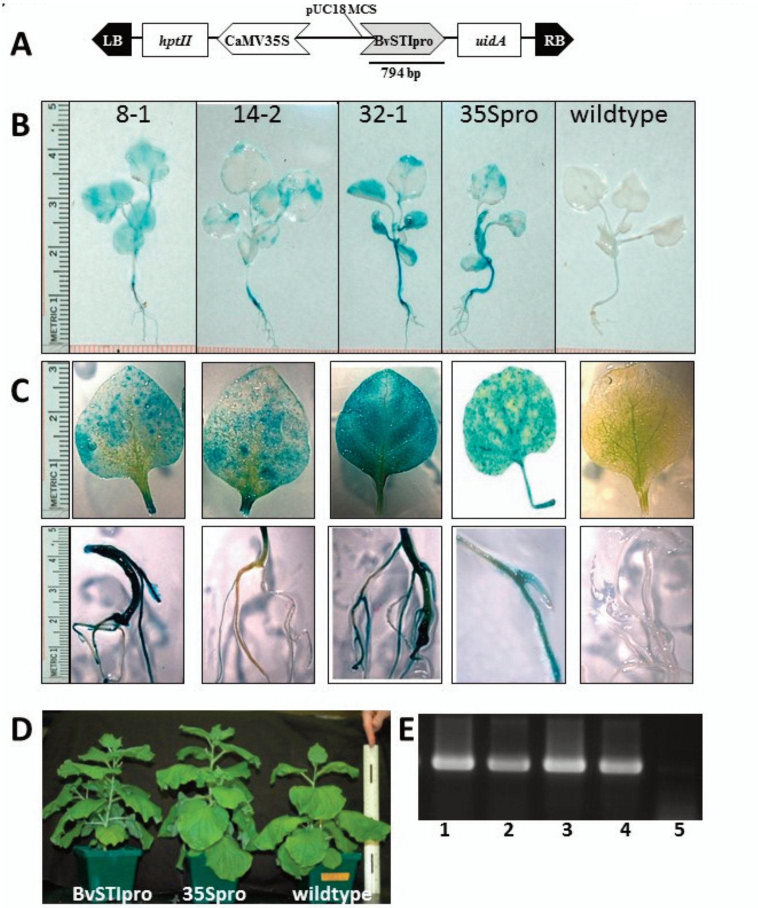

Fig. 1 A) Schematic representation of the sugar beet BvSTI promoter fused to the GUS (uidA) gene, BvSTIpro-GUS, with nopaline synthase PolyA terminator in plant expression vector pCAMBIA 1301. Left (LB) and right (RB) T-DNA borders; hygromycin phosphotransferase (hptII) selectable marker gene under control of Cauliflower mosaic virus 35S promoter (CaMV35S); multiple cloning site (pUC18MCS).
B) Histochemical localization of GUS activity in 2-week-old seedlings of BvSTIpro-GUS (BvSTIpro 8-1, 14-2, 32-1), CaMV35S-GUS (35Spro) and untransformed wildtype plant; C) leaves and roots of 6-week-old plants in B). D) Transgenic BvSTIpro, 35Spro and wildtype plant. E) RT-PCR analysis of leaves from transgenic and normal wildtype plants; lane 1-3, BvSTIpro-GUS 8-1, 14-2, 32-1, respectively, lane 4, 35Spro, lane 5 untransformed wildtype plant. DNA band corresponds to a 544 basepair GUS gene fragment.

The pCAMBIA 1301 vector with *GUS* gene fused to the CaMV 35S constitutive promoter was used as a positive control for transformation (35S-*GUS*). The PlantCARE database (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/; Lescot et al., 2002) was used to analyze the cloned 794 bp sequence for any *cis*-acting motifs in the *BvSTI* regulatory region.

Nicotiana benthamiana transformation

Agrobacterium tumefaciens EHA 105 strain harboring either the BvSTIpro-*GUS* or 35S-*GUS* gene was co-cultivated with tobacco (*Nicotiana benthamiana* Domin) leaf explants (1 cm^2) excised from fully expanded leaves of greenhouse-grown plants using previously reported methods (Smigocki et al., 2013). Briefly, sterilized explants were immersed in bacterial suspensions for 10 min, blotted dry on sterile filter paper and placed on MS media (Murashige and Skoog 1962) with B5 vitamins (Gamborg et al., 1965), 3 % sucrose and 0.7 % agar. After 2 days of co-cultivation in the dark at 25 °C, explants were washed with sterile solutions of cefotaxime and carbenicillin (500 mg/l each) and placed on callus-induction medium (CIM: MS salts, B5 vitamins, 6-benzylaminopurine (BAP) 2 mg/l, 200 mg/l cefotaxime and 500 mg/l carbenicillin). Shoots regenerated from derived calli were excised and cultured on 1/2 MS selection medium (SM) containing BAP 0.5 mg/l and Hyg 20 mg/l. Shoots were transferred to rooting medium (RM: 1/2 MS medium with no hormones, supplemented with Hyg 20 mg/l). Rooted shoots were acclimated, transferred to a growth chamber (25 ± 2 °C day, 22 ± 2 °C night and 16/8 h light conditions, respectively) and then to a greenhouse (25 ± 5 °C day, 22 ± 3 °C night, 16/8 h light conditions). Normal untransformed *N. benthamiana* plants were used as negative controls. Seeds collected from the regenerated plants (T0) were germinated on 1/2 MS salts with Hyg (40 mg/l) to select Hyg resistant T1 plants from seeds that segregated at a 3:1 ratio of resistant to susceptible plants. Based on the number of resistant and susceptible plants, the expected segregation ratio for each T1 line was tested using the chi-square (χ^2) test (Greenwood and Nikulin 1996).

PCR and RT-PCR analysis of BvSTIpro-*GUS* transformed plants

Genomic DNA was purified from regenerated plants (Haymes 1996) and analyzed by PCR. *GUS* gene specific primers used to amplify a 544 bp fragment from the genomic DNA were: Forward 5'ATGGTAGATCTGAGGAACC3' and Reverse 5'GTTCACACAAACGGTGATAC3'. PCR conditions were: 35 cycles at 95 °C (1 min), 53 °C (1 min), 72 °C (2 min) and one cycle at 72 °C (10 min). PCR products were analyzed by electrophoresis on 1.2 % agarose gels.

Total RNA was isolated using RNeasy Plant Mini Kit (Qiagen, USA) from approximately 100 mg of fresh leaf tissue and treated with RNase-free DNase (Qiagen, USA). Titanium One-Step RT-PCR Kit (Clontech Laboratories Inc., CA, USA) was used to amplify the *GUS* gene transcript from about 100 ng of RNA under the following conditions: 50°C (1h), 94°C (2 min 40 sec), 30 cycles of 94°C (30 sec), 60°C (40 sec), 72°C (1 min 30 sec), followed by 72°C (5 min), using the same primers as described above. RT-PCR results were normalized with the constitutively expressed plant actin gene primers: Forward 5'-GTATTGTKAGCAACTGGGATGA-3' and Reverse 5'-AACKYTCAGCCCRATGGTAAT-3') under the same conditions as above. RT-PCR analyses were repeated two times.

Histochemical analysis of *GUS* expression

Histochemical β-glucuronidase (GUS) assays were used to determine *GUS* gene expression in transformed plant tissues (Jefferson et al., (1987). X-Gluc (5-bromo-4-chloro-3-indolyl β-D-glucuronide; 0.5 mg/ml) was the substrate for the β-glucuronidase enzyme encoded by the *GUS* gene. Stained tissues were examined and photographed with a Stereo Discovery V20, Zeiss microscope. (Carl Zeiss Microscopy, LLC, Thornwood, NY)

Wound-induced *GUS* expression

Six leaves were excised from mid-sections of 14-week-old BvSTIpro-*GUS*, 35S-*GUS* and control plants. Three leaves were wounded by making three 1 cm long incisions on each side of the leaf, avoiding the mid-veins, and three were used as the non-wounded control. After 6 and 24 h, leaves were analyzed for GUS activity. For analysis of *GUS* expression in the roots, roots were excised at the hypocotyl and washed under running cold water. The root mass was then divided into sections that were mechanically wounded by pinching with forceps at 5 mm intervals over the entire root length. One root section was used as an unwounded control. Roots were stained for *GUS* expression after 6 and 24 h. Experiments were repeated 3 times.

For insect wounding experiments, one second instar fall armyworm (FAW; *Spodoptera frugiperda* J.E. Smith) was used to infest an excised leaf or roots of BvSTIpro-*GUS*, 35S-*GUS* and control plants. Newly emerged FAW larvae were purchased from Benzon Research (Carlisle, PA) and reared on artificial diet provided by the supplier. Insects were maintained at room temperature for 1 to 3 days and removed from the diet 2 h prior to start of experiments. Weighed second instars were placed on an excised leaf or roots and incubated on water saturated filter paper in Petri dishes. After feeding, remaining leaves and roots were analyzed for *GUS* gene activity. Experiments were repeated 3 times.

RESULTS

Sequence analysis of the cloned *BvSTI* regulatory region

The 794 bp 5' *BvSTI* regulatory region was analyzed for the presence of *cis*-regulatory elements (Table 1). Using the PlantCARE database, multiple putative *cis*-elements were found. These included

Table 1. The putative *cis*-acting elements and functions predicted by PlantCARE in the *BvSTI* regulatory sequence.

Name of the element	**(Strand)**	**Sequence**	**Function**
3-AF1 binding site	(-)	AAGAGATATTT	light responsive element
ARE	(-)	TGGTTT	*cis*-acting regulatory element essential for the anaerobic induction
ATCT-motif	(-)	AATCTAATCC	part of a conserved DNA module involved in light responsiveness
Box 4	(+)	ATTAAT	part of a conserved DNA module involved in light responsiveness
CATT-motif	(+)	GCATTC	part of a light responsive element
E-box	(+)	CANNTG	*cis*-acting element involved in defense and stress responsiveness
HD-Zip 1	(+)	CAAT(A/T)ATTG	*cis*-acting element involved in defense and stress responsiveness and in differentiation of the palisade mesophyll cells
HD-Zip 2	(+)	CAAT(G/C)ATTG	*cis*-acting element involved in the control of leaf morphology development
HSE	(+)	AAAAAATTTC	*cis*-acting element involved in heat stress responsiveness
MNF1	(+)	GTGCCC(A/T)(A/T)	*cis*-acting element involved in light responsiveness
Skn-1_motif	(+)	GTCAT	*cis*-acting regulatory element required for endosperm expression
TC-rich repeats	(+)	ATTTTCTCCA	*cis*-acting element involved in defense and stress responsiveness
TCT-motif	(+)	TCTTAC	part of a light responsive element
TCT-motif	(+)	TCTTAC	part of a light responsive element

the expected CAAT and TATA boxes, as well as several putative stress-response *cis*-elements, in both the sense and antisense strands. The following stress elements were found: a CANNTG sequence, known as the E-box, CAAT(A/T)ATTG known as the HD-Zip1, and ATTTCCTCCA , a TC-rich repeat element (Meshi and Iwabuchi 1995; Henriksson et al., 2005; Cabello et al. 2012). Another identified stress sequence known as the HSE element, AAAAAATTTC, is known to function as a binding site for heat shock transcription factors (Gao et al. 2008).

Transgenic *N. benthamiana* BvSTIpro-*GUS* plants

Rooted tobacco shoots that regenerated on hygromycin-containing selection media were analyzed by PCR for the presence of the *GUS* gene. Several PCR positive plants were identified and subsequently stained for GUS activity to confirm expression of the *GUS* gene from the BvSTIpro regulatory sequence. All selected GUS positive BvSTIpro-*GUS* plants exhibited normal growth patterns and phenotypes like normal, untransformed control plants (Fig. 1D). Seeds collected from six independently derived T0 GUS positive BvSTIpro-*GUS* plants germinated on Hyg-containing media to generate T1 plants 3-1, 3-4, 8-1, 14-2, 31-1 and 32-1. The segregation ratio of 3:1 hygromycin resistant to susceptible seedlings fit the Mendelian ratios for a single locus insertion of the *hpt*II gene in the T1 generation of plants. A chi-square test revealed no statistical differences between observed and expected segregation of hygromycin resistance and susceptibility for all tested lines indicating that the plants were hemizygous for the *hpt*II gene which was inherited as a single dominant allele. Screening of the T2 seeds for hygromycin resistance revealed that seeds collected from independently derived T1 lines 3-4 and 8-1 were homozygous for the expression of the *hpt*II gene, i.e. 100 % of the T2 seeds grew on Hyg-containing media. RT-PCR analysis of leaf RNAs from T1 BvSTIpro-*GUS* (8-1, 14-2, 32-1) and 35S-*GUS* (35S) plants showed the expected 544 bp *GUS* gene DNA fragment (Fig. 1E).

GUS expression in BvSTIpro-*GUS* plants

Transformed BvSTIpro-*GUS* lines 8-1, 14-2 and 32-1 were analyzed for GUS activity at various developmental stages from seedling to mature plants. GUS activity was detected in leaves and roots of 2- and 6-week-old seedlings (Fig. 1B and C). The level of expression was similar to that observed in the constitutive 35S-GUS positive control (35S), with one exception. BvSTIpro-*GUS* transformant 32-1 exhibited GUS activity that was relatively higher than the degree of GUS staining detected in the other transformants and the constitutively expressed 35S-GUS plants. Transformant 14-2 showed the weakest level of *GUS* expression in 2-week old leaves and in 6-week-old roots. When mature plants that were 10 to 14-weeks old were analyzed, *GUS*

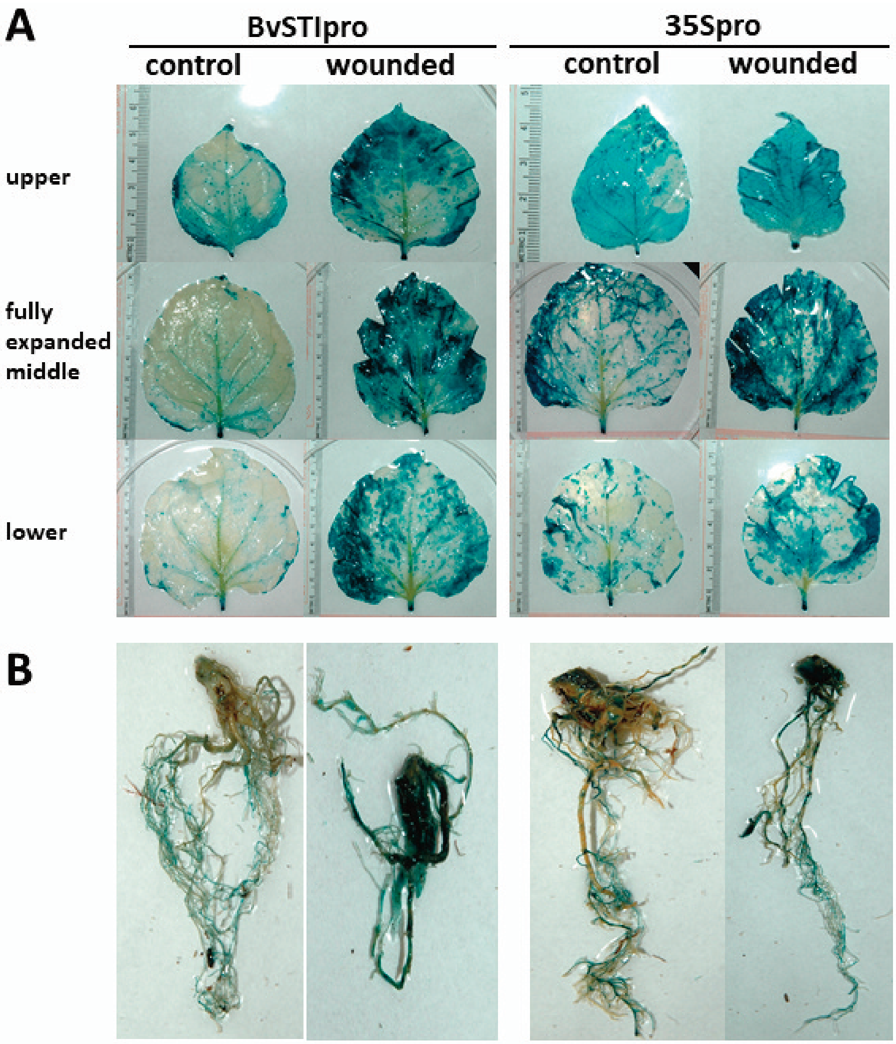

Fig. 2 Histochemical localization of GUS activity in leaves and roots of 14-week-old transgenic BvSTIpro (8-1) and 35Spro plants. A) GUS activity 6 hours following mechanical wounding of leaves compared to non-wounded leaves (control). Upper are younger leaves that represent the upper one third of the plant, fully expanded middle, the middle third, and lower, the bottom third leaves on the plant. B) GUS activity following mechanical wounding of roots compared to non-wounded (control) roots as in A).

expression in leaves and roots of BvSTIpro-*GUS* plants was reduced as compared to the younger 2- and 6-week old plants and the 35S-GUS positive controls (BvSTIpro control in Fig. 2, 3 and 4).

Wound induced GUS expression in leaves and roots

Physical damage incited by mechanical wounding of leaf edges increased GUS activity levels in mature 14- week old BvSTIpro-GUS plants (Fig. 2A). GUS activity increased in all leaves that included young (top third of stem height), fully expanded (middle third of stem) and mature (bottom third of stem) leaves. The activity increased throughout the entire leaf and was not confined to the incision sites. The increased GUS levels were comparable relative to the GUS activity detected in the 35S-GUS leaves. The up-regulation of GUS activity in the BvSTIpro-GUS leaves was evident at 6 and 24 (data not shown) h after wounding (Fig. 2A, BvSTIpro wounded). No significant GUS activity increase due to wounding of the 35S-GUS control was observed, except for what seemed to be a slight increase in the fully expanded leaves. Wounding of BvSTIpro-GUS roots increased the level of GUS activity throughout the root mass at 6 and 24 (data not shown) h after wounding (Fig. 2B). Root expression in the 35S-GUS positive control transformant remained relatively high before and after wounding. No GUS activity was noted in the untransformed wildtype leaves and roots prior to or after wounding (Fig. 1B, 1C, 3 and 4).

Insect induced *GUS* expression

Leaves and roots from mature 14-week old transgenic BvSTIpro-*GUS* plants 8-1, 14-2 and 32-1 were analyzed for GUS activity following infestation with FAW larvae (Fig. 3 and 4). Wounding incited by larval feeding increased GUS activity above the basal levels (control) in both leaves and roots of BvSTIpro-*GUS* plants. At 24 h after infestation, GUS activity was detected throughout the infested leaves and roots at similar levels to those in 35S-*GUS* plants. Leaves and roots from normal, wildtype plants exhibited similar levels of damage incited by the feeding larvae with no detectable GUS activity (Fig. 3 and 4).

DISCUSSION

Present study focused on the temporal, spatial and inducible expression patterns of a regulatory sequence that was cloned from the sugar beet *BvSTI* PI gene. *BvSTI* was shown to be a defense gene with a demonstrated role in insect pest resistance (Puthoff and Smigocki 2007; Smigocki et al., 2008; Smigocki et al., 2013). In sugar beet, *BvSTI* transcripts were shown to accumulate to higher levels in plant varieties that are resistant to the sugar beet root maggot as compared to the susceptible germplasm (Puthoff and Smigocki 2007; Savić and Smigocki 2012). In addition, *BvSTI* gene expression in sugar beet was up-regulated by FAW larval feeding and mechanical wounding (Savić and Smigocki 2012). To analyze the potential use of the *BvSTI* regulatory

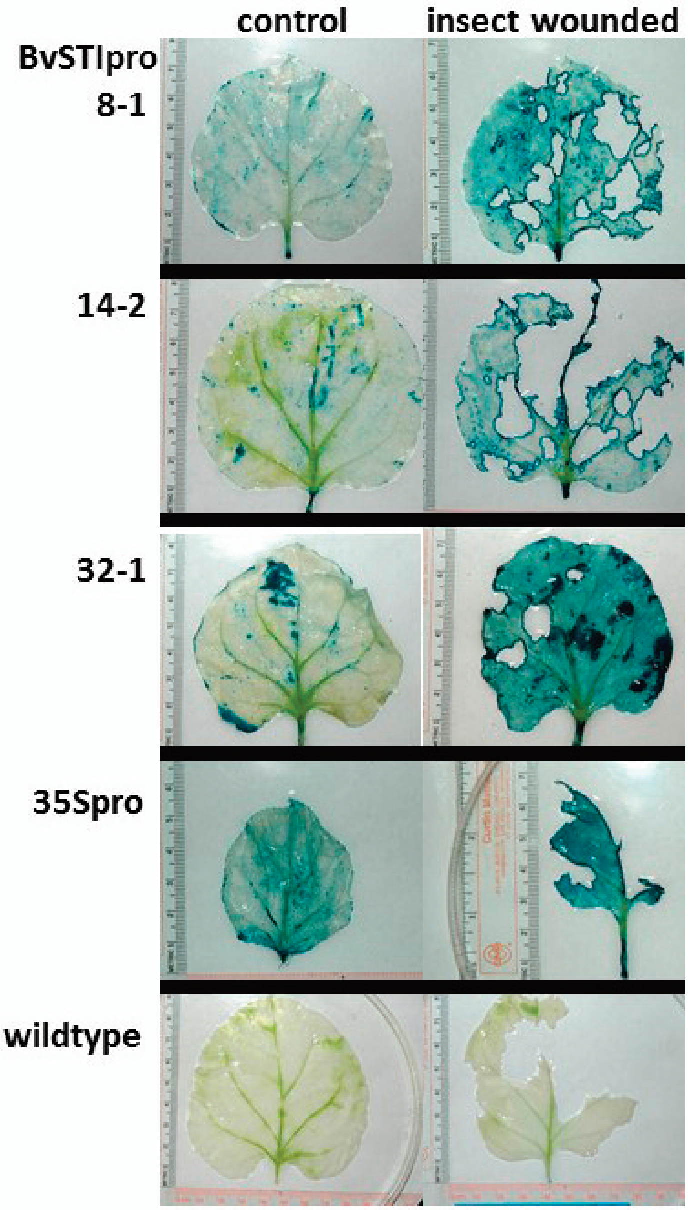

Fig. 3 GUS activity induced by FAW feeding on BvSTIpro-*GUS* leaves of transformant 8-1, 14-2 and 32-1 and 35S-GUS (35Spro) at 24 hours. Wildtype leaves are from untransformed plants. Leaves were excised from the middle third of 14-week old plants.

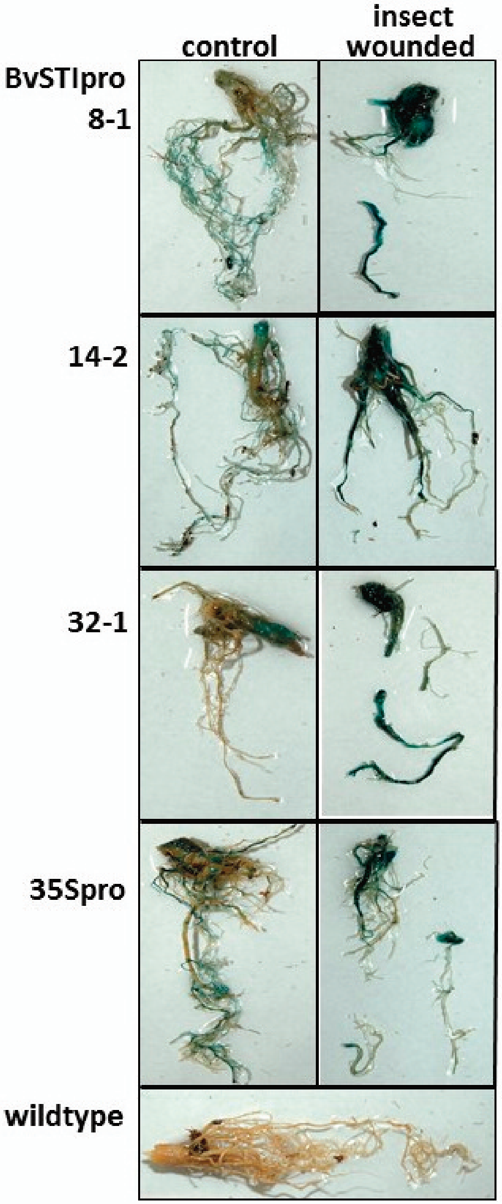

Fig. 4 GUS activity induced by FAW feeding on BvSTIpro-*GUS* roots of transformant 8-1, 14-2 and 32-1 and 35S-GUS (35Spro) at 24 hours. Wildtype roots are from untransformed control plants. All roots were excised from 14-week old plants.

region for directing the expression of beneficial transgenes in a heterologous plant system, we transformed *N. benthamiana* plants with a chimeric vector in which the expression of the *GUS* reporter gene was placed under the control of the *BvSTI* gene regulatory sequence (BvSTIpro-*GUS*).

Developmental and stress induced regulation of *GUS* gene expression by the BvSTI regulatory sequence was observed in BvSTIpro-*GUS* transgenic tobacco plants. The level of expression in leaves and roots of transgenic plants varied depending on the age of the plant. In younger plants that were 2- and 6-weeks old, relatively high levels of GUS activity were detected throughout the leaf and root that were comparable to the expected high levels of expression from the constitutive 35S promoter (Fig. 1B and C). As the plants aged, GUS levels were significantly reduced. In maturing 14-week old plants, relatively low basal levels of GUS activity were detected in the leaves and roots of the BvSTIpro plants as compared to the 35Spro plants that over-express the GUS reporter gene (Fig. 2A, Fig. 3 and 4 control). However, analysis of several leaves at varies stages of growth, i.e. younger leaves from the upper third of the plant, fully expanded leaves from the middle third of the plant, and lower fully expanded older leaves, showed a similar pattern of developmental regulation of GUS activity (Fig. 2A, control). The highest GUS activity was visualized in the younger upper leaves as in the 2- and 6-week old plants. Increasingly reduced GUS levels were observed in the middle leaves followed by the oldest leaves. This trend was more significant in the BvSTIpro plants than in the 35S-GUS plants suggesting that the BvSTI promoter is developmentally regulated. However, it is also likely, that at least in small part, the reduced GUS activity in the older fully expanded leaves is due to the physiological stage of the leaf as it enters senescence. Root GUS activity also appeared to be somewhat lower in the mature plants as compared to roots from 2- and 6-week old plants, but this trend was less consistent (Fig. 1C, 2B; Fig. 4 control). The most interesting outcome, however, is the documented induction of GUS activity in the mature plants by abiotic and biotic stresses. Physical damage of the BvSTIpro-GUS leaves in mature plants, irrespective of their developmental stage, induced highly up-regulated GUS activity in the upper, middle and lower leaves. Similar induction was detected in the roots. Wounding, whether mechanical or by insect feeding, increased GUS activity levels which were similar to those observed in the 35S-GUS plants (Fig. 2, wounded; Fig. 3, Fig. 4). This suggests that the BvSTI promoter should prove useful for driving the expression of resistance genes that potentially could protect plants at all developmental stages, starting with constitutive expression in the younger plants and being highly inducible as the plants mature.

Similar developmental regulation of PI gene expression in plants has been reported (Ryan 2000). The potato PI *pinII* gene was shown to be constitutively expressed in flowers and tubers and was wound inducible in leaves (Keil et al., 1989; Pena-Cortes et al., 1991). Although a wound inducible characteristic is highly desirable for a regulatable promoter, it is uncommon for a promoter to have strictly controlled expression limitations between constitutive and inducible expression. Certain levels of GUS activity were always detected in the BvSTIpro-*GUS* plants at all developmental stages. However, wounding damage of mature plants induced an even higher level of GUS expression in leaves and roots of older plants. A similar expression pattern was reported for the poplar *win3.12* promoter in potato roots in response to mechanical wounding and fungal infection (Yevtushenko et al., 2004). The reported constitutive and inducible expression patterns suggest that the genes driven by these promoters may be involved in the preparation of the tissue for defense, especially to target the large number and variety of pests and pathogens known to afflict the roots.

The constitutive regulation of the *BvSTI* promoter in the transgenic *N. benthamina* plants during the early stages of development (at 2 and 6 weeks) may be attributed to a physiological process associated with these stages of growth. Regulation, for example, by yet undefined transcription factors, may induce higher levels of gene expression from the BvSTI promoter in younger plants. However, one cannot rule out that the promoter may be hypersensitive to the effects of the physical environment in which the plantlets grow and develop. The impact of tiny water droplets from a mister was sufficient to induce GUS activity in *Arabidopsis* leaves transformed with an inducible proteinase inhibitor (*pinII*) gene promoter that was fused to the *GUS* gene (Godard et al., 2007). Also *in vitro* conditions of high humidity, low irradiation, supraoptimal levels of macronutrients and sugar have been shown to be stress factors that induce defense responses, especially in young sensitive plantlets (Desjardins et al., 2009). Snyder et al. (1999) reported that in tissue cultured plants where GUS activity was driven by the *osm* gene promoter, GUS activity was tenfold higher than that observed with the constitutive 35S promoter. However, when the *osm-GUS* plants were grown in the greenhouse, expression of the osm-*GUS* gene was found to be inducible but not constitutive.

In agriculture, the use of pest and pathogen inducible promoters offers the advantage of increasing tolerance and resistance while minimizing the potential negative effects on plant growth that are often associated with constitutive overexpression of resistance transgenes. These classes of promoters are valuable tools for genetic modification of crops because they specifically direct targeted expression to the site of pest introgression and pathogen infection. The wound- and stress-inducible characteristics of PI promoters make them exceptionally valuable for production of recombinant insecticidal and bactericidal proteins and compounds when the plant is attacked by an invading pest or pathogen. In the case of the *BvSTI* promoter, GUS activity in mature

14-week-old *N. benthamiana* leaves and roots was for the most part relatively low under normal growth conditions. But the increase in gene activity in response to mechanical or insect wounding was significant for expression of beneficial resistance genes needed for the plant to combat the onset of disease development. The constitutive leaf and root expression at the early developmental stage is valuable for enhancing resistance in younger, more vulnerable plantlets. Therefore, these results suggest that incorporating defense related transgenes under the control of the *BvSTI* gene regulatory sequence should benefit the plant's ability to defend itself against invading pests and pathogens during its growth and development.

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