A *BETA VULGARIS* SERINE PROTEINASE INHIBITOR GENE (*BvSTI*) REGULATED BY SUGAR BEET ROOT MAGGOT FEEDING ON MODERATELY RESISTANT F1016 ROOTS

A. C. Smigocki, D. P. Puthoff, S. D. Ivic-Haymes and S. Zuzga
USDA-ARS, Molecular Plant Pathology Laboratory, BARC-West B004,
10300 Baltimore Ave., Beltsville, MD 20705

Introduction

The sugar beet root maggot (SBRM, *Tetanops myopaeformis*) is one of the most devastating insect pests of sugar beet that is found in two-thirds of all U. S. sugar beet fields and accounts for 10-100% reduction in yields (Mahrt and Blickenstaff 1979; Yun 1986). Larval feeding causes deformed root structure and secondary pathogen invasion, both contributing to the reduced yield and product quality. Ineffective cultural practices and harmful chemical insecticides are the only available measures for control of the root maggot; therefore, a strong impetus exists for development of effective and environmentally friendly control measures. No completely resistant germplasm has been identified and the only currently available lines reduce the SBRM damage ratings by approximately 40% (Campbell et al. 2000).

To explore novel strategies for management of SBRM and gain knowledge of root defense response mechanisms, we examined root gene expression incited by SBRM in a moderately resistant F1016 and a susceptible F1010 line (Puthoff and Smigocki, 2007). We identified more than 150 sugar beet root ESTs responding to infestations by SBRM using the suppressive subtractive hybridization method and RT-PCR confirmation. The largest number of identified ESTs from both the susceptible and resistant genotype grouped into the defense and stress response classes. The other ESTs included genes involved in secondary metabolism and signal transduction. Gene expression profiles of the cloned genes were also obtained using macroarrays following mechanical wounding and treatment of roots with methyl jasmonate, salicylic acid and ethylene. Of the examined root ESTs, the greatest number were regulated by methyl jasmonate and salicylic acid suggesting these signaling pathways may be involved in sugar beet root defense responses to SBRM. A gene of particular interest, coding for a serine (trypsin) protease inhibitor (*BvSTI*), was identified in the F1016 EST library. *BvSTI* shares sequence similarity with a tomato gene (*LeMir*) that is primarily expressed in the maturing epidermis of the root, is induced by invading nematodes and is secreted to the rhizosphere (Brenner et al., 1998). Given serine proteases comprise the major digestive enzymes in root maggot midguts (Wilhite et al. 2000), our findings suggest that *BvSTI* may be involved in the resistance mechanism of F1016. To elucidate the functional role of *BvSTI* in insect resistance and root biology, the *BvSTI* gene was reconstructed for over-expression and used to transform sugar beet.

Materials and Methods

Plant material

Plants of the sugar beet breeding lines F1010 (Campbell, 1992) and F1016 (Campbell et al. 2000) were grown in the greenhouse at 25 ^{0}C (day) and 22 ^{0}C (night) under natural light.

SSH and Differential Hybridization

Suppressive subtractive hybridization (SSH) was conducted using the PCR-Select cDNA Subtraction Kit (BD Biosciences, www.bdbiosciences.com) as described in manufacturer's instructions with 2 µg polyA$^+$ RNA (Puthoff and Smigocki, 2007). Three complete subtractions were conducted: 1) F1010 SBRM infested vs. uninfested, 2) F1016 SBRM infested vs. uninfested and 3) F1010 vs. F1016 with both uninfested and SBRM infested tissues.

Sequencing and BLAST

Sequencing of differentially expressed clones was carried out at the DNA Synthesis and Sequencing Facility, Iowa State University, Ames, IA. Raw sequences were subjected to batch BLASTX (Altschul et al. 1997) analysis against the GenBank non-redundant database. Batch BLASTN was also conducted against the TIGR *Beta vulgaris* gene index (BvGI) to identify sugar beet ESTs. Individual clones were compared to each other, using local BLASTN, to identify a unique set of ESTs.

RNA isolation and RT-PCR

Total RNA was isolated as previously described (Puthoff and Smigocki, 2007). For RT-PCR, RNA from the same tissues used for SSH was DNase treated (DNA-free, Ambion). First strand cDNA was synthesized using SuperScript II (Invitrogen) as directed by the manufacturer. Equal amounts of cDNA template (10 ng) were used in each PCR reaction using annealing temperature specific for each gene.

BvSTI gene constructs

To obtain the full length coding sequence of *BvSTI* from the cloned EST, 5' and 3' RACE kits (BD Biosciences, San Jose, CA) were used with gene specific primers. After both 5' and 3' RACE clones were sequenced, gene specific primers were designed and used to amplify the entire *BvSTI* coding region. *BvSTI* was fused to the 35S promoter in the pCAMBIA 1301 plant transformation vector (pBvSTICDS). To clone the *BvSTI* promoter, a PCR-based strategy was employed (Universal GenomeWalking Kit, BD Biosciences, San Jose, CA). Following sequence verification, the BvSTI promoter gene fragment was fused with the *uidA* (*GUS*) gene in the pCAMBIA 1301 plant transformation vector (pBvSTIpro).

Plant transformation

Agrobacterium rhizogenes strain 15834 carrying the pBvSTICDS or pBvSTIpro transformation vector was grown overnight and then resuspended in liquid half strength (1/2) B5 medium (Gamborg *et al.*, 1968). Petiole transformation was essentially performed as described by Kifle et al. (1999). Petioles were excised from fully expanded leaves of greenhouse-grown plants and surface-sterilized. Petioles were then cut into 1 cm long pieces and infected with the *A. rhizogenes* strains for 10 min, blotted dry and plated on 1/2 B5 medium. After 2d of co-

cultivation in the dark at 25^{0}C, explants were washed with Cefotaxime and carbenicillin (500 mg/l each) and plated on 1/2 B5 medium containing 250 mg/l of the above antibiotics. Regenerated hairy roots were excised and cultured on 1/2 B5 medium containing 5 mg/l hygromycin.

Histochemical analysis of GUS expression

GUS activity was assayed by incubating the hairy roots in an X-Gluc (5-bromo-4 chloro-3-indolyl-b-*d*-glucuronic acid) solution at 37 ^{0}C overnight (Jefferson et al. 1987).

***BvSTI* proteinase inhibitor activity assays**

Soluble proteins were extracted with 1 mM HCl and used in a radial diffusion assay (Broadway and Missurelli, 1990; Jongsma et al., 1993). Proteins were quantified (Bradford, 1976; Bio-Rad) and immediately used in the assay using bovine trypsin (0.001 mg/ml, Sigma) as substrate. Proteinase activity was visualized by incubating the gel with a substrate-dye solution of 2.5 mg/ml N-acetyl-DL-phenylalanine β-naphthyl ester (Sigma) and 0.5 mg/ml tetrazotized O-dianisidine (Sigma) at 37°C for 30 min. The diameter of the clear zones was measured and the proteinase activity quantified using purified soybean proteinase inhibitor (Fluka) as standard.

For analysis of trypsin inhibitory activity in polyacrylamide gels (Chan and De Lumex, 1982; Wang et al. 2003), native proteins were extracted in 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 10 mM EDTA, 10% sucrose, 10 mM ascorbic acid, 1 mM PMSF and 2 mM DTT. Protein concentrations were determined as described above and 5-10 μg of total proteins were separated on 12% native polyacrylamide gels. After electrophoresis and removal of SDS, proteins were re-natured and incubated with the bovine trypsin substrate. Acetic acid (10% v/v) was added to stop the reaction and clear zones corresponding to trypsin inhibitory activity were noted.

Fall Armyworm Bioassay

Fall armyworm (*Spodoptera frugiperda*) eggs were hatched on sugar beet leaves. Newly hatched larvae were used to develop a feeding bioassay with the transformed hairy roots.

Results and Discussion

The SSH libraries we generated are enriched for genes important in the initial responses of sugar beet roots to insect herbivory. Our experimental system utilized tissues from a feeding bioassay capable of screening for SBRM resistance and thus reflects field-like conditions (Smigocki et al., 2006). While not definitive, the degree of sequence similarity of the ESTs at the amino acid level can aid in the identification of protein function and give a starting point for determination of a gene's role in plant cells. Functional annotation grouped the unique clones into many different categories (Figure 1). However, the largest number of clones fell into the defense-related class reported to be regulated by other pathogens including insect pests.

In general, plant defenses are thought to be controlled by three major signaling molecules: jasmonates, salicylic acid and ethylene. These signaling pathways cross-talk with each other in order to respond appropriately to each pathogen (see review Feys and Parker 2000). Methyl jasmonate treatment of F1016 sugar beet roots altered the expression of 65 (35 up-regulated; 30 down-regulated) of more than 160 ESTs we cloned (Puthoff and Smigocki, 2007). Similarly, salicylic acid regulated 32 genes (29 up-regulated; 3 down-regulated) and ethylene altered levels of 18 of the transcripts (17 up-regulated; 1 down-regulated). Exact gene sequence

matches were not found when comparing F1010-regulated genes to F1016-regulated genes; however, common putative functions were identified. A closer examination of the respective genes would shed light on whether or not they are the same genes or members of a family that respond differently to environmental stimuli (Velazhahan et al. 1998; Wang and Constabel 2004).

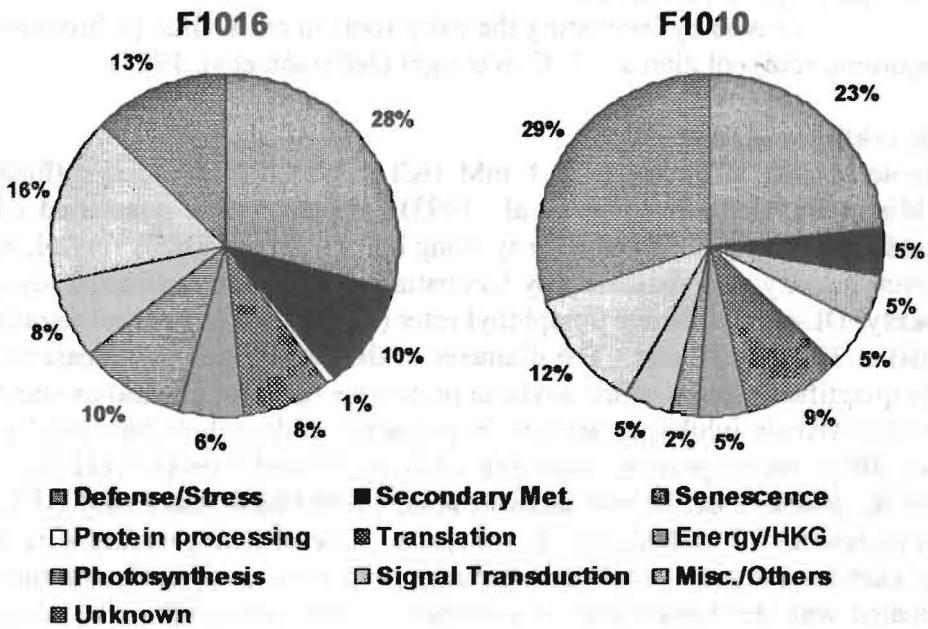

Figure 1. Functional groups of sugar beet genes identified by SSH as responding to SBRM infestation. The percentage of ESTs falling into each category is shown. The total number of genes identified was 121 for F1016 and 42 for F1010.

Because SBRM larvae feed with a scraping mechanism, we expected up-regulation of wound response genes such as proteinase inhibitors, lipoxygenases and phenylalanine ammonia lyase in our collection of SBRM-responsive genes. However, only one protease inhibitor clone (*BvSTI*) was identified that shares sequence similarity with a class of serine proteinase inhibitors (Jofuku and Goldberg, 1989; Brenner et al. 1998). Regulated expression of *BvSTI* is associated only with the moderately resistant F1016 sugar beet genotype (Puthoff and Smigocki, unpublished). Interestingly, we have previously shown that serine protease activity was one of the major digestive activities in SBRM larval midguts (Wilhite et al. 2000) leading us to speculate that the cloned *BvSTI* serine proteinase inhibitor gene may play a role in SBRM resistance.

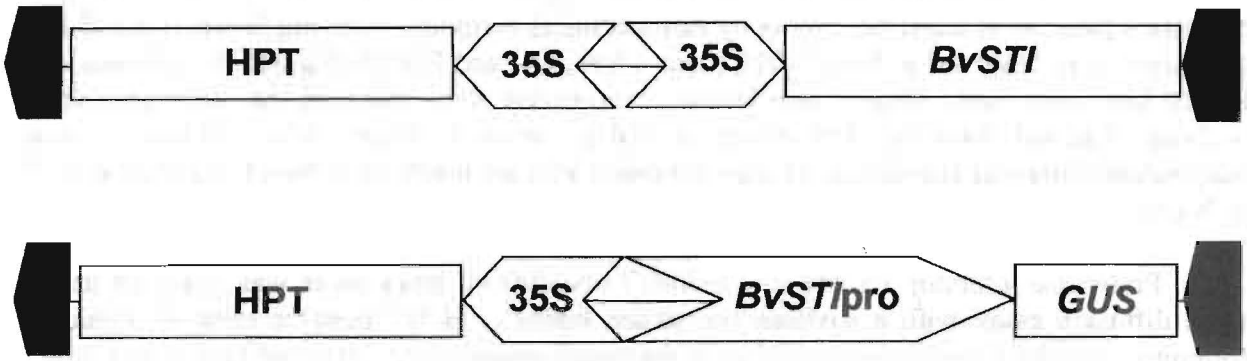

Figure 2. The *BvSTI* coding region was fused to the 35S promoter and the $BvSTI_{pro}$ was fused to the GUS gene in pCAMBIA 1301 transformation vector with HPT (hygromycin neomycin phosphotransferase) gene as a selectable marker.

BvSTI shares sequence similarity with a tomato gene (*LeMir*) that is primarily expressed in the maturing epidermis of the root, is induced by invading nematodes and is secreted to the rhizosphere (Brenner et al., 1998). To elucidate the functional role of *BvSTI* in insect resistance and root biology, the *BvSTI* gene and its promoter were cloned and reconstructed for analysis of expression in sugar beet hairy roots (Figure 2).

A number of independently transformed sugar beet hairy root lines carrying the *BvSTI* gene or the *GUS* gene driven by the *BvSTI* gene promoter were regenerated from both the SBRM susceptible F1010 and moderately resistant F1016 genotypes (Figure 3). Hairy roots carrying *BvSTI* driven by the constitutive 35S promoter showed a variable pattern of root growth, which ranged from slow to fast (Figure 3A). Preliminary analyses indicated that the rate of hairy root growth was not inversely proportional to the level of *BvSTI* gene expression, i.e. slow growth, high levels of expression (data not shown).

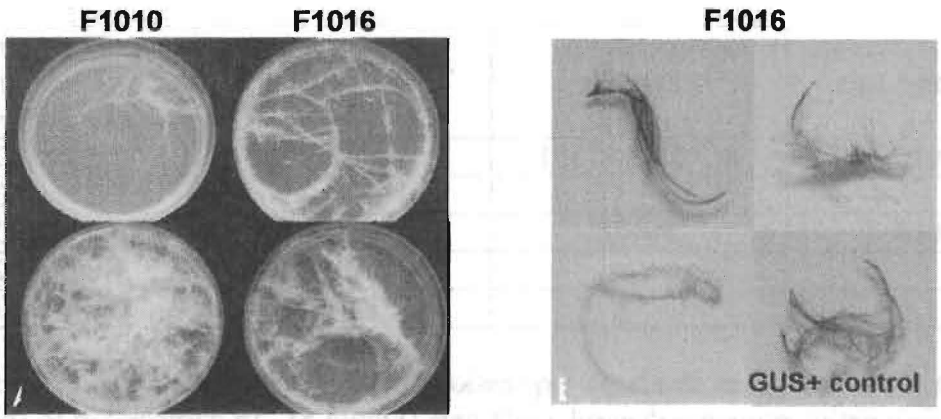

Figure 3. A) F1010 and F1016 hairy roots transformed with *BvSTI* after 3 weeks in culture. B) BvSTI promoter-GUS gene expression in F1016 hairy roots.

Fusion of the *BvSTI* promoter to the GUS gene was used to determine *BvSTI* gene expression patterns in sugar beet roots by histochemical methods. Varying levels of GUS gene expression were observed in both F1010 (data not shown) and F1016 (Figure 3B) independently derived hairy root lines. Constitutive levels of expression were observed that were comparable to those observed with the 35S promoter (GUS+ control, Figure 3B). However, many independently derived transformants also exhibited varying levels of reduced expression of the GUS gene from the *BvSTI* promoter.

Proteinase inhibitor activity in the *BvSTI* transformed hairy roots was analyzed using a radial diffusion assay with a soybean proteinase inhibitor as the positive control (Figure 4). Preliminary results indicate that a number of the transformants (202, 207) had higher levels of activity when compared to hairy roots transformed with the empty vector. Proteinase inhibitor activity ranged from about 0.04 to almost 0.07 µg TI equivalent/µg protein in the *BvSTI* lines as compared to 0.02 in the empty pCAMBIA 1301 vector control (Table 1).

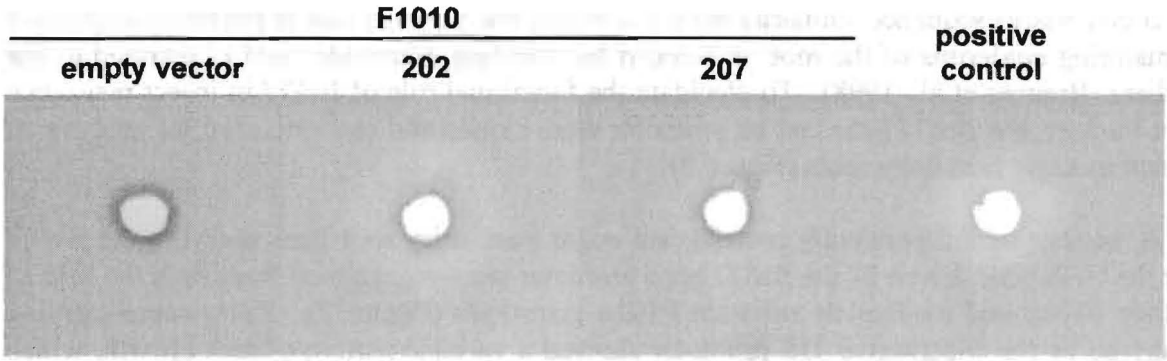

Figure 4. BvSTI proteinase inhibitor activity in transformed sugar beet hairy roots (empty vector – negative control, 202, 207) analyzed using a radial diffusion assay and trypsin as the substrate. Soybean trypsin inhibitor - positive control.

Table 1. Average trypsin inhibitory (TI) activity in F1010 hairy roots transformed with the *BvSTI* gene or the pCAMBIA 1301 empty vector (control) determined by radial diffusion assay.

Sugar beet line	Construct	Transformed line	TI activity [µg TI equivalent/ µg protein
F1010	pCAMBIA1301	1	0.021±0.005
		2	0.020±0.012
F1010	*BvSTI*	202	0.067±0.031
		207	0.048±0.015
		211	0.041±0.005

Lines that showed highest levels of expression of the *BvSTI* gene were subjected to further analysis using native protein polyacrylamide gels (Figure 5). In comparison to the control roots (Figure 5, lane 1-4), new proteinase inhibitor activity was detected in only the *BvSTI* transformed roots (lane 5-8). To confirm that the new proteinase inhibitor activity observed in the

transformed roots corresponds to the BvSTI protein, we are in the process of analyzing the transformants by Western blots with BvSTI-specific antibody.

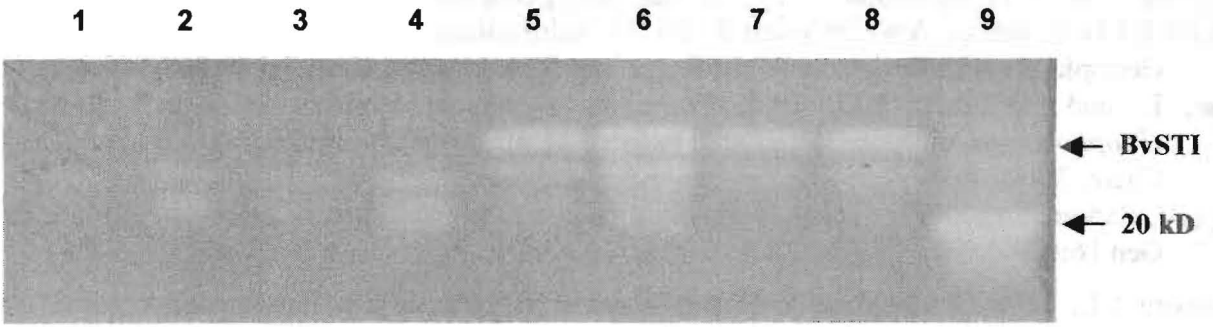

Figure 5. Analysis of BvSTI activity in transformed sugar beet hairy roots following protein separation in a 12% PAGE using trypsin as substrate. Lane 1 - 4, F1010 untransformed control; 5 - 8, F1010 BvSTI transformant 202 and 207, in duplicate, respectively; 9, positive control (soybean Kunitz trypsin inhibitor protein, 20 kDa, 1.5 µg). Total protein: 5 µg in 1, 3, 5 and 7 and 10 µg in 2, 4, 6 and 8.

BvSTI transformed hairy root lines showing high levels of proteinase inhibitor activity will be used in an *in vitro* SBRM bioassay (Smigocki et al., 2006) to determine the effect of the *BvSTI* gene on larval feeding. In addition, bioassays are being developed to test the effect of *BvSTI* on other pests of sugar beet such as the fall armyworm that are known to utilize serine proteases as their major digestive enzymes. Preliminary data indicates that newly hatched fall armyworms will feed on transformed sugar beet hairy roots *in vitro* (Figure 6).

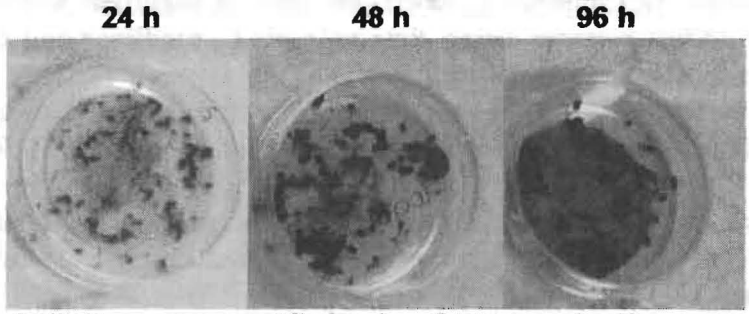

Figure 6. Fall armyworm (*S. frugiperda*) larvae feeding on sugar beet hairy roots.

Refecences

Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 25:3389-3402.

Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72: 248–254.

Brenner ED, Lambert KN, Kaloshian I and Williamson VM (1998) Characterization of *LeMir*, a Root-Knot Nematode-Induced Gene in Tomato with an Encoded Product Secreted from the Root. Plant Physiol 118:237–247.

Broadway, R. M., and Missurelli, E. L. 1990. Regulatory mechanisms of tryptic inhibitory activity in cabbage plants. Phytochemistry 29: 3721–3725.

Campbell LG (1992) Registration of F1010 sugar beet germplasm. Crop Sci. 30:.429-430.

Campbell LG, Anderson AW, Dregseth RJ (2000) Registration of F1015 and F1016 Sugar beet Germplasms with Resistance to the Sugar beet Root Maggot. Crop Sci 40:867-868.

Chan, L. and DeLumex, B.O. 1982. Properties of trypsin inhibitor from winged bean (*Psophocarpus tetragonolonus*) seed isolated by affinity chromotography. J. Agric. Food Chem. 30: 42–46.

Feys BJ, Parker JE (2000) Interplay of signaling pathways in plant disease resistance. Trends in Gen 16:449-455.

Gamborg UL, Mille, RA, Ojima K (1968) Nutrient requirements of suspension cultures of soybean root cells. Exp Cell Res 50: 48-51.

Jefferson RA, Kavanagh TA, Bevan MW (1987) GUS fusions: β-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J 6: 3901-3907.

Jongsma, M. A., Baker, P. L., and Stiekema, W. J. 1993. Quantitative determination of serine proteinase inhibitor activity using a radial diffusion assay. Anal. Biochem. 212: 79–84.

Jofuku, K. D., & Goldberg, R. B. (1989). Kunitz trypsin inhibitor genes are differentially expressed during the soybean life cycle and in transformed tobacco plants. *The Plant cell, 1*(11), 1079-1093.

Kifle S, Shao M, Jung C, Cai D (1999) An improved transformation protocol for studying gene expression in hairy roots of sugar beet (*Beta vulgaris* L.). Plant Cell Rep 18: 514-519.

Mahrt CG, Blickenstaff CC (1979) Host plants of the sugar beet root maggot, *Tetanops myopaeformis*. Ann Entomol Soc Am 72:627-631.

Smigocki AS, Ivic-Haymes S, Puthoff D, Campbell L, Boetel M. 2006. A sugarbeet root maggot (*Tetanops myopaeformis* Röder) bioassay using *Beta vulgaris* L. seedlings and in vitro propagated transformed hairy roots. J Sugar Beet Res 43:1-13.

Puthoff, D. P. and Smigocki, A. C. (2007) Insect feeding-induced differential expression of *Beta vulgaris* root genes and their regulation by defense-associated signals. Plant Cell Reports 26:71-84.

Velazhahan R, Chen-Cole K, Anuratha CS, Muthukrishnan S (1998) Induction of thaumatin-like proteins (TLPs) in *Rhizoctonia solani*-infected rice and characterization of two new cDNA clones. Physiol Plant 102:21-28.

Wang H-Y., Huang Y-C., Chen S-F., Yeh K-W. 2003. Molecular cloning, characterization and gene expression of a water deficiency and chilling induced proteinase inhibitor I gene family from sweet potato (Ipomoea batatas Lam.) leaves. Plant Science 165:191-203.

Wang J, Constabel CP (2004) Three polyphenol oxidases from hybrid Poplar are differentially expressed during development and after wounding and elicitor treatment. Physiol Plant 122:344-353.

Wilhite SE, Elden TC, Puizdar V, Armstrong S, Smigocki AC (2000) Inhibition of aspartyl and serine proteinases in the midgut of sugar beet root maggot with proteinase inhibitors. Entomol Exp Appl 97:229-233.

Yun YM (1986) Part II. Major insects and arthropods. In: Whitney ED, Duffus JE (eds) Compendium of Beet Diseases and Insects. APS Press, St. Paul, pp 40-50.