MOLECULAR APPROACHES FOR CONTROL OF THE SUGARBEET ROOT MAGGOT

ANN SMIGOCKI[1*], SNEZANA D. IVIC[1], DENNIS WILSON[1], CHRIS A. WOZNIAK[2], LARRY CAMPBELL[3], ROBERT DREGSETH[4] AND MARK BOETEL[4]

[1]*USDA, ARS, Molecular Plant Pathology Laboratory, Beltsville MD 20705,*
[2] *Biopesticide and Pollution Prevention Division, EPA, Washington, DC 20460*
[3]*USDA, ARS, Northern Crop Science Laboratory, Fargo, ND 58105,*
[4]*North Dakota State University, Department of Entomology, Fargo, ND 58105*

ABSTRACT

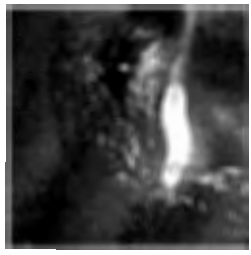
The sugarbeet root maggot (*Tetanops myopaeformis* Roder) is a major insect pest of sugarbeet in the United States and Canada and accounts for yield losses ranging from 10 to 100%. Currently no biological control measures exist and crop rotation is ineffective due to the mobility of the adult flies and existence of several weed species as substitute hosts. A few insecticides are available but provide inconsistent results. In the last few years, we have developed a method for direct gene transfer to sugarbeet leaves that uses greenhouse-grown plants and generates transgenic plants within three months. We have identified and engineered a number of beneficial genes for specific expression in sugarbeet leaves and taproots. One class of genes targets the digestive system of the maggot thus starving the insect. We identified two major classes of digestive enzymes in midguts excised from feeding maggots and demonstrated their inhibition by specific proteinase inhibitors. Genes encoding these proteinase inhibitors will be introduced into sugarbeet to evaluate their effect *in planta*. We have also initiated studies to profile the defense response genes in maggot resistant sugarbeet lines. As a first step, we developed an *in vitro* root maggot bioassay using resistant (F1016) and susceptible parental (F1010) lines to generate infested tissues as source of mRNA for preparation of differential cDNA libraries enriched for resistance genes. Clones with potential roles in root maggot and disease resistance will be characterized, reconstructed for plant expression and their role in resistance evaluated in transgenic plants.

INTRODUCTION

Disease and insect pest problems have had a significant negative impact on sugar production from sugarbeet. The sugarbeet root maggot (SBRM), *Tetanops myopaeformis* Roder, was first described as a sugarbeet pest in Utah in the 1920's. It is now considered a major pest of sugarbeet in the United States and Canada where greater than half or all of the sugarbeet acreage, repectively, is infested with the root maggot. This Dipteran inflicts yield losses that can range from 10 to 100% in infested fields (Cooke 1993). Developing larvae feed on tap and feeder roots throughout the growing season causing damage either by severing the taproots of seedlings or badly scarring the

surface of larger roots (Figure 1). Damaged taproots are predisposed to diseases caused by opportunistic pathogens such as *Erwinia carotovora* subsp. *betavsculorum*, *Aphanomyces cochlioides*, and *Rhizoctonia solani*. Granular insecticides of the carbamate or organophosphate classes are often used to reduce larval populations in sugarbeet fields, although control is inconsistent. In addition, many of these pesticides are being re-evaluated as mandated by the Food Quality

Figure 1. *A first instar root maggot feeding on a developing taproot of a sugarbeet seedling.*



Protection Act of 1996 and may be removed from the list of approved insecticides, leaving few alternatives. Crop rotation practices have been ineffective mainly due to the mobility of the adult flies. Existence of several weed species as substitute hosts has also hindered population control. No effective biological control measures are currently on the market. A biocontrol fungus, *Synglіocladium tetanopsis*, has been patented as a naturally occurring pathogen of SBRM and is currently in the process of evaluation and development (Hodge et al., 1998; Wozniak, 1999). Similarly, two entomopathogenic fungi, *Beauveria bassiana* and *Metarhizium anisopliae*, have shown promise in preliminary screenings and field trials (Campbell et al., 2000b). The lack of effective control measures that do not rely on broad-spectrum insecticides has hastened the search for environmentally friendly alternative strategies.

Protection of plants from herbivorous insect pests has traditionally relied on conventional breeding programs for incorporation of resistance traits. With the advent of molecular biology, insect resistance genes have been identified, cloned and transferred to heterologous plants to impart disease resistance. Gene transfer technology is an economical and environmentally favorable approach to reduce the usage of toxic chemicals for insect control. The most widely exploited approaches have utilized plants that were genetically modified with resistance genes that target an insect's digestive system. Promising results have already been achieved with commercially available transgenic plants expressing modified delta-endotoxin genes (Bt) from the soil bacterium *Bacillus thuringiensis*. However, since many insect pests are not susceptible to the Bt toxin, novel insecticidal genes need to be identified and evaluated for applicability of this approach to pest control.

Molecular approaches to enhance disease and insect resistance in sugarbeet have been hampered by a general lack of a reliable gene transfer method, a small pool of well characterized defense genes, and knowledge of sugarbeet

defense responses. Our efforts are focused on several approaches geared towards the development of effective strategies for the control of SBRM (Table 1). One of the approaches involves the development of genetically modified sugarbeets that express proteinase inhibitor (PI) genes to specifically target midgut proteases of SBRM larvae. By blocking the major classes of digestive proteases in actively feeding maggots, the assimilation of nutrients from ingested foods would be inhibited and thus thwart the normal growth and development of the insect (Wilhite et al., 2000). Another approach involves the manipulation of the production of toxic compounds *in planta*. These compounds are mainly products of secondary metabolic pathways, many of which have been shown to play a role in plant defense responses. Our efforts are also aimed at characterizing sugarbeet defense response mechanisms. Profiling of genes in resistant sugarbeet lines is an approach that will provide useful information for developing new control strategies for the root maggot and other pests.

Table 1. *Molecular approaches for control of the sugarbeet root maggot.*

- Development of sugarbeet gene transfer methods
- Cytokinin-induced insecticidal compounds
  - Modulation of secondary metabolism
  - Cytochrome P450 genes
- Inhibition of digestive proteases in larval midguts
  - Proteinase inhibitor genes
- Identification of insect/disease resistance genes in SBRM resistant lines
  - sugarbeet defense response mechanisms

1.- SUGARBEET TRANSFORMATION

In the last few years, we developed and optimized a number of sugarbeet transformation methods in order to improve the efficiency with which beneficial genes can be introduced into the sugarbeet genome (Ivic and Smigocki, 2001; Ivic and Smigocki, 2003a; 2003b; Ivic et al., 2001a; 2001b; Snyder et al., 1999). In general, we found that the sugarbeet methods in the public domain are not readily reproducible and yield low transformation frequencies. These methods utilize sugarbeet cotyledons, shoot basal tissues, and hypocotyl callus. Therefore, as a first step for developing a reliable transformation protocol for commercially important sugarbeet lines, we optimized the production of highly embryogenic cells for use with the particle bombardment gene transfer method. Using leaves of greenhouse-grown sugarbeet breeding lines, we determined which plants consistently produced highly regenerative leaf callus. From this callus, we prepared short-term suspension cultures and bombarded the cells with the *uidA* (GUS) reporter and the *npt* II selectable marker genes. Transformed calli and embryos were detected by histochemical stains for GUS gene expression and stable incorporation of the GUS gene into the sugarbeet genome was confirmed by Southern blot analyses. The main advantage of using short-term suspension cultures is that the method is less time consuming and labor intensive than the particle bombardment protocol that was developed for hypocotyl callus of a clonal, tissue culture line Rel1 (Snyder et al., 1999).

We also tested the possibility that the leaves of plants that generated the highly embryogenic callus could be used directly for particle bombardment. The elimination of the tissue culture step required to produce the embryogenic leaf callus and suspension cultures shortened the time for regeneration of transformed plants to about 3 months after bombardment (Ivic and Smigocki, 2003a). The efficiency of transformation, calculated as the number of shoots expressing GUS per number of bombarded leaf fragments, ranged from 0.9% to 3.7%. The advantages of this transformation method include an abundant source of leaf material from greenhouse-grown plants, the ease of handling leaf material in tissue culture, and the overall rapid regeneration of transgenic shoots.

2.- PLANT-DERIVED INSECTICIDAL COMPOUNDS

We are exploring the potential use of plant-derived insecticidal compounds for pest control. We discovered that *Nicotiana* plants transformed with the cytokinin biosynthesis gene *ipt* had elevated levels of cytokinins that were correlated with an acquired insect resistance (Smigocki et al. 1993). As cytokinin applications have been linked to the accumulation of secondary metabolites, many of which have insecticidal properties, our studies with transgenic plants that overproduce cytokinin also suggest the involvement of cytokinins in the modulation of secondary metabolic pathways. We demonstrated that most of the insecticidal activity was recovered in methylene dichloride surface extracts from transgenic *ipt* plants (Smigocki et al. 1997; Smigocki et al. 2000). The activity was stable for longer than 1 year when stored at 4 C in the dark. A number of insects representing different insect orders were susceptible to the extracts. Tobacco hornworm (Lepidoptera), sugarbeet root maggot (Diptera), and green peach aphid (Homoptera) were either killed or their normal development and reproduction were severely affected (Smigocki et al. 1993; Smigocki et al., 2003). Exposure of sugarbeet root maggot larvae to the extracts induced an almost immediate twitching and thrashing behavioral response that was followed by death. We observed that more than 90% of the larvae died after 5 days of exposure to a 1% suspension of the extract (Table 2). These results suggest that cytokinin-mediated insect resistance could be an effective strategy for control of the sugarbeet root maggot.

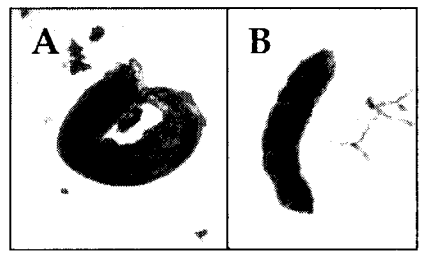
Table 2. *Effects of surface extracts from transgenic ipt and untransformed N. plumbaginifolia plants on first-instar SBRM after 1 and 5 days of exposure.*

		Mortality (%)		Twitching (%)[1]	
		Day			
		1	5	1	5
Extract/Concentration					
Transgenic *ipt*	1%	0	92	68	42
	0.1 %	0	83	10	40
Untransformed	1%	0	23	0	19
	0.1%	0	53	0	20
Saline control		0	26	0	0

[1]*Percent of live larvae that were twitching.*

Purification of the *Nicotiana* insecticidal extracts has not proceeded to where biological activity could be ascribed to any one or more of the compounds. Chemical analyses of partially purified extracts have revealed compounds suggestive of oxygen-containing aliphatic molecules in the molecular weight range of diterpenes (Smigocki, et al. 1997; Smigocki et al. 2000). Terpenoids play diverse functions in plants that include a role as herbivore repellents. Exposure of SBRM larvae to a suspension of the partially purified fraction of the extract induced a similar twitching and thrashing response that was followed by death as was observed with the unfractionated extract (Figure 2).

Figure 2. *A) First-instar SBRM exposed to a partially purified fraction of the cytokinin-induced insecticidal compounds. B) Larva in saline.*



Identification of the active compounds in the *Nicotiana* extracts will help define how cytokinins modulate their production, secretion or availability and lead to additional biotechnological approaches for environmentally friendly insect control. As a step towards a better understanding of how cytokinins modulate plant defense mechanisms, we are analyzing cytokinin-induced gene regulation. From a *Nicotiana plumbaginifolia* cDNA library enriched for cytokinin-responsive genes, we identified over 100 cDNAs that are up-regulated by cytokinin (Harding and Smigocki, 1994). Included in that set is a gene that codes for a cytochrome P450 monooxygenase, *CYP*72A2 (Mujer and Smigocki, 2001). Plant cytochrome P450s are heme-containing enzymes that participate in the synthesis of a wide variety of secondary metabolites, some of which are inhibitory to the survival of pathogens and insects. The *CYP*72A2 gene has sequence homology to a gene that is involved in synthesis of a number of pharmaceuticals and insecticidal compounds. We demonstrated that wounding stress and feeding insects systemically induce the expression of *CYP*72A2. The induction is more rapid in transgenic plants that overproduce cytokinin and in response to insect damage. Our initial plant transformation experiments with a constitutively expressed *CYP72A2* gene produced no tobacco or tomato transformants but numerous *N. plumbaginifolia* transformants were obtained (Bartoszewski et al., 2002; Smigocki, unpublished). These results suggest that in heterologous plant systems, *CYP72A2* gene expression may need to be stringently regulated to reduce a build up of toxic levels of secondary metabolites that would cause cell death.

Metabolic engineering of plants via genetic modification of the P450 enzymes has powerful implications for molecular farming for natural plant chemicals used as pharmaceuticals and disease and insect deterrents. Although the pathways for the synthesis of secondary metabolites are complex and involve numerous enzymatic reactions, fortuitously, they appear to be similar in all crop plants so strategies are being devised that will require only limited modifications for

increased production of a potentially useful metabolite.

3.- PROTEINASE INHIBITORS FOR INSECT CONTROL

A class of genes that code for proteinase inhibitors (PIs) have been shown to enhance insect resistance in experimental trials (Delledonne et al., 2001; Duan et al., 2001; Maqbool et al., 2001; Samac and Smigocki, 2002). Selected PIs specifically target insect digestive proteases that release essential nutrients from ingested foods. Normal growth and development of the insect depends on this process and, therefore, has been exploited as a target for insect control. Inhibition of the digestive enzymes could potentially starve the larvae as there is a heavy reliance among insects on endoproteinases for the purpose of assimilating dietary protein.

To devise a rational control strategy, it is necessary to first determine the particular digestive enzymes utilized by a specific pest as significant variations have been found in the types and properties of digestive enzymes utilized by insects (Terra & Ferreira, 1994). Therefore, in order to determine which PIs might be effective against SBRM, we characterized the major midgut proteases in feeding second instars collected from infested fields in Minnesota (Wilhite et al., 2000). We determined that there are three predominant classes of protease activity in SBRM midgut extracts (Table 3).

Two components of the activity were evident at an acidic pH with an optimum of 2.5 or lower, and another had a pH optimum of approximately 8.5. Low-molecular weight biochemical inhibitors that target the major mechanistic classes of insect digestive endoproteinases were used to determine the nature of the proteases in the SBRM extract (Table 3). We demonstrated that Pepstatin A with preferential specificity toward aspartyl proteases was by far the most effective inhibitor at an acidic pH (84% inhibition). PMSF which targets serine proteases reduced proteolysis in SBRM extracts by 50%. E-64, which has high potency toward virtually all known cysteine proteinases, had a minor inhibitory activity of about 7%.

We also tested the effect of several plant-derived PIs on the proteolytic activity (Table 3). Squash aspartyl proteinase inhibitor blocked virtually all the proteolytic activity, confirming the importance of the aspartyl class at acidic pH. Soybean trypsin-chymotrypsin inhibitor (Bowman-Birk I) blocked nearly all proteolysis at pH 8.5, suggesting the presence of trypsin and/or chymotrypsin-like serine proteases in the extract. Similarly, rice oryzacystatin I that targets cysteine proteases blocked approximately 20% of the activity.

Earlier studies have shown that when fed a particular PI in their diets, insects compensated for the inhibition by producing more proteases that are insensitive to that PI. However, despite the compensation, insects suffered considerable negative effects from the presence of the PI (Jongsma et al., 1996). A combination of PIs in the insect diets was found to be more toxic at levels where individual inhibitors were not effective. Similarly, higher levels of more than one PI were found in plants that were resistant vs. susceptible to a particular insect (Oppert et al., 1993; Piergovanni et al., 1991). These findings suggest that transformation of plants with more than one class of PI genes to target the proteolytic activities in the insect gut will likely prove to be the most effective and

sustained means of controlling insect infestations. We identified PI genes with specificity for the aspartyl, serine, and cysteine class of proteases in SBRM midguts that will be introduced into sugarbeet for evaluation of their effect on SBRM larvae.

Table 3. Digestive proteases in SBRM midguts and class specific proteinase inhibitors that block their activity.

SBRM Midgut Proteases (pH)	Biochemical Inhibitors (% inhibition)	Plant PIs (% inhibition)
Aspartyl 2.5	Peptstatin A (84)	Squash aspartyl (80)
Serine 8.5	PMSF (50)	Bowman Birk I (95)
Cysteine 2.5	E64 (7)	Oryzacystatin I (20)

4.- DEFENSE GENE PROFILING

Natural plant defense mechanisms have been under-exploited in part due to a lack of a thorough understanding of the numerous complex and integrated defense responses. To effectively manipulate the plant/pest or plant/microbe interactions, a better understanding of both the genetics and biochemistry of resistance is needed. Gene expression profiling provides new insights into groups of genes whose expression is altered during the interactions of the microbe or insect with the host and for the discovery of cellular genes that were not previously recognized as being regulated by infection or infestation. In addition, analysis of these genes provides knowledge of the regulatory mechanisms that govern plant gene expression and will lead to the development of technologies for controlling gene expression to achieve inherent increased productivity and quality characteristics such as insect/disease resistance in plant germplasm.

We have initiated studies to characterize the defense response genes of sugarbeet, especially in the sucrose storing taproots that are prone to attack by numerous pests and pathogens. Two breeding lines, F1016 and F1015, have been released as SBRM resistant germplasm (Campbell et al., 2000a). Using these lines and one of the parental lines, F1010, as a control, we developed a root maggot bioassay for generating infested tissues enriched for resistance genes (Figure 1). Infested tissues were collected at three time points within 48 hr of when larvae were first placed on sugarbeet seedlings and, cDNA libraries were prepared from the mRNA extracted from these tissues. The libraries are being screened with a subtracted probe enriched for genes associated with resistance. Subtracted probes are prepared by hybridization of mRNA (cDNA) from the infested, resistant line with a 5-fold excess of mRNA from the infested, susceptible line to remove RNA transcripts that are found in both the resistant and susceptible plants. We anticipate that this approach will lead to the identification of sugarbeet clones with potential roles in root maggot and disease resistance. These genes will be characterized and reconstructed for targeted

and regulated over- or under-expression in order to analyze their participation in plant defense mechanisms.

In addition, we plan to prepare a microarray panel of sugarbeet genes using the putative resistance-associated cDNA clones and approximately a few thousand additional cDNAs from the libraries. The microarray panels will be analyzed by simultaneous hybridization with probes prepared from the mRNA from infested resistant and susceptible lines, each labeled with a different fluorescent dye. This approach will yield information about groups of genes whose expression is altered during the interaction of the root maggot with the sugarbeet taproot and lead to the discovery of new genes induced by insect infestation.

CONCLUSION

In our efforts to improve the disease resistance of sugarbeet using genetic engineering approaches, we developed a number of gene transfer methods employing particle bombardment of tissues that we first established to be highly embryogenic. The advantages of the optimized methods include the use of leaf material from an abundant source of greenhouse-grown plants and the overall rapid regeneration of genetically modified plants. We conceived several strategies for possible control of SBRM, the most devastating insect pest of sugarbeet. We determined that there are three primary digestive proteases in SBRM midguts. These proteases were effectively blocked with plant-derived PIs, suggesting that genetic modification of sugarbeet with PI genes should impart maggot resistance. We also showed that cytokinin-induced insecticidal compounds effectively killed SBRM larvae leading us to believe that the genetic manipulation of the production of these toxic compounds *in planta* may be an effective control strategy for the maggot. The gene profiling studies in root maggot-resistant lines have progressed to the point of screening of the cDNA libraries for genes associated with the defense response. We fully anticipate that this approach will provide useful information for developing new, environmentally friendly strategies for control of the root maggot and other pests.

REFERENCES

1. BARTOSZEWSKI, G., MUJER, C. V., NIEMIROWICZ-SZCZYTT, K. & SMIGOCKI, A.: Cloning of a wound inducible *Lycopersicon esculentum* cytochrome P450 gene and lack of generation of transgenic plants with sense or antisense constructs. *J. Am. Soc. Hort. Sci.* 127:535-539, 2002.

2. CAMPBELL, L. G., ANDERSON, A. W. & DREGSETH, R. J.: Registration of F1015 and F1016 sugarbeet germplasms with resistance to the sugarbeet root maggot. *Crop Sci.* 40:867-868, 2000a.

3. CAMPBELL, L. G., EIDE, J. D., SMITH, L.J. & SMITH, G. A.: Control of the sugarbeet root maggot with the fungus *Metarhizium anisopliae*. *J. Sugar Beet Res.* 37:57-69, 2000b.

4. COOKE, D. A.: Pests. *In*: D. A. Cooke & R. K. Scott (eds.), The Sugar Beet Crop. Chapman and Hall; London, pp. 428-483, 1993.

5. DELLEDONNE, M., ALLEGRO, G., BELENGHI, B., BALESTRAZZI, A., PICCO, F., LEVINE, A., ZELASCO, S., CALLIGARI, P. & CONFALONIERI, M.: Transformation of white poplar (*Populus alba* L.) with a novel *Arabidopsis thaliana* cysteine proteinase inhibitor and analysis of insect pest resistance. *Mol. Breed.* 7:35-42, 2001.

6. DUAN, X. L., LI, X. G., XUE, Q. Z., ABO-EL-SAAD, M., XU, D. & WU, R.: Transgenic rice plants harboring an introduced potato proteinase inhibitor II gene are insect resistant. *Nature Biotechnol.* 14:494-498, 1996.

7. HARDING, S. A. & SMIGOCKI, A.: Cytokinins modulate stress response in *ipt*-transformed *Nicotiana plumbaginifolia* plants. *Physiol. Plantarum* 90:327-333, 1994.

8. HODGE, K. T., HUMBER, R. A. & WOZNIAK, C. A.: *Cordyceps variabilis* and the genus *Synglíocladium*. Mycologia 90(5): 745-753, 1998.

9. IVIC, S. & SMIGOCKI, A.: Evaluation of the biolistic transformation method for commercially important sugarbeet lines. *Proc. Am. Soc. Sugar Beet Technologists*:207-213, 2001.

10. IVIC, S. & SMIGOCKI, A.: Direct gene transfer to sugarbeet leaves. Advances in Sugar Beet Research, 5: 2003a.

11. IVIC, S. & SMIGOCKI, A.: Transformation of sugarbeet cell suspension cultures. *In Vitro Cell. & Develop. Biol. – Plant*, submitted, 2003b.

12. IVIC, S., SAUNDERS, J. W. & SMIGOCKI, A.: Leaf callus from sugarbeet breeding lines for biolostic transformation. *Proc. Am. Soc. Sugar Beet Technologists*:214-216, 2001a.

13. IVIC, S., SICHER, R. C., & SMIGOCKI, A.: Growth habit and sugar accumulation in sugarbeet (*Beta vulgaris* L) transformed with a cytokinin biosynthesis gene. *Plant Cell Reports* 20:770-773, 2001b.

14. JONGSMA, M., STIEKEMA, W.J. & BOSCH, D.: Combating inhibitor-insensitive proteases of insects. *Trends in Biotechnology* 14:331-333, 1996.

15. MAQBOOL, S. B., RIAZUDDIN, S., LOC, N. T., GATEHOUSE, A. M. R., GATEHOUSE, J. A. & CHRISTOU, P.: Expression of multiple insecticidal genes confers broad resistance against a range of different rice pests. *Mol. Breed.* 7:85-93, 2001.

16. MUJER, C. V. & SMIGOCKI, A. C.: Wound-inducible cytochrome P450 from *Nicotiana plumbaginifolia*. *Physiol Plant* 111:172-181, 2001.

17. OPPERT, B., MORGAN, T., CUBERTSON, C. & KRAMER, K.: Dietary mixtures of cysteine and serine proteinase inhibitors exhibit synergistic toxicity toward the red flour beetle, *Tribolium castaneum*, *Comp. Biochem. Physiol.* 105C:379-385, 1993.

18. PIERGOVANNI, A., SONNANTE, A. G., GATTA, C. & PERRINO, P.: Digestive enzyme inhibitors and storage pest resistance in cowpea (*Vigna unguiculata*) seeds. *Euphytica* 54:191-194, 1991.

19. SAMAC, D. A. & SMIGOCKI, A. C.: Expression of oryzacystatin I and oryzacystatin II in alfalfa increases resistance to the root-lesion nematode (*Pratylenchus penetans*). *Phytopathology* 91: in press, 2003.

20. SMIGOCKI, A., NEAL, J. W. JR., MCCANNA, I. & DOUGLASS, L.: Cytokinin-mediated insect resistance in Nicotiana plants transformed with the ipt gene. *Plant Mol. Biol.* 23:325-335, 1993.

21. SMIGOCKI, A. C., CAMPBELL, L. G. & WOZNIAK, C. A.: Leaf extracts from cytokinin-overproducing transgenic plants kill sugarbeet root maggot (*Tetanops myopaeformis*) larvae. *J. Sugar Beet Res.*, submitted, 2003.

22. SMIGOCKI, A. C., HEU, S. & BUTA, G.: Analysis of insecticidal activity in transgenic plants carrying the ipt plant growth hormone gene. *ACTA Physiol. Plant* 22:295-299, 2000.

23. SMIGOCKI, A. C., HEU, S., MCCANNA, I., WOZNIAK, C. A. & BUTA, G.: Insecticidal compounds induced by regulated overproduction of cytokinins in transgenic plants. In *Advances in Insect Control, The Role of Transgenic Plants*, edited by N. Carozzi and M. Koziel. Bristol: Taylor and Francis, Ltd., 1997.

24. SNYDER, G. W., INGERSOLL, J., SMIGOCKI, A. C. & OWENS, L. D.: Introduction of novel pathogen and defense genes and a cytokinin biosynthesis gene into sugarbeet (*Beta vulgaris* L.) by particle bombardment of *Agrobacterium*. *Plant Cell Reports* 18:829-834, 1999.

25. TERRA, W. R. & FERREIRA, C.: Insect digestive enzymes: properties, compartmentalization and function. *Comp. Biochem. Physiol.* 109B:1-62, 1994

26. WILHITE, S. E., ELDEN, T. C., PUIZDAR, V., ARMSTRONG, S. & SMIGOCKI, A.C.: Inhibition of aspartyl and serine proteinases in the midgut of sugarbeet root maggot with proteinase inhibitors. *Entomol exp appl.* 2000.

27. WOZNIAK, C. A.: Novel fungal pathogen for biocontrol of the sugarbeet root maggot. USA Patent #5,955,071, 1999.