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Effects on the Development of *Plutella xylostella* and *Spodoptera litura* after Feeding on Transgenic Cabbage Expressing Potato Proteinase Inhibitor II and *Bar* Genes

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Abstract

Cabbage plants were transformed with the potato proteinase inhibitor II (PINII) gene, *bar* gene, and *hpt* gene using *Agrobacterium*. The expression of the PINII gene was driven by its own promoter which was wound-inducible. Ten transgenic plants were obtained from medium containing hygromycin as a selection antibiotic. The integration and expression of PINII and *bar* genes were confirmed by Southern and Northern hybridization. Growth and development of diamondback moths (*Plutella xylostella*) and tobacco cutworm (*Spodoptera litura*) larvae were examined on T_1 plants. The weight of the larvae and pupae of these two insects grown on transgenic plants was not different compared to those grown on wild type plants. However, the pupation and emergence rate of diamondback moths and tobacco cutworms fed on some transgenic plants was lower than on wild type plants. These results suggest that the PINII transgene under the control of a wound-induced promoter may be used for control of insects in transgenic cabbage through reduction of insect progeny number.

Key words: *Agrobacterium*, cabbage, transgenic plant, proteinase inhibitor

Introduction

Insect predation is a major factor in the reduction of crop yield. Engineering crops with insecticidal protein genes could reduce the cost, time and effort spent protecting crops from insect pests and contribute to an environmentally friendly agricultural system.

Production of transgenic plants resistant to insects is an attractive way to control plant pests. Remarkable results were obtained by engineering plants with insecticidal crystal protein genes from *Bacillus thuringensis* (Bt). A number of *Brassica* species have been engineered with various *Bt* endotoxin genes (Ding et al. 1998; Cao et al. 1999; Cho et al. 2001). Other approaches using plant derived proteins or genes also have been developed. For example, anti-metabolic proteins synthesized in plants interfere with the digestive process in insects. These proteins are produced constitutively in plants and can be induced by mechanical wounding and insect attack. In many cases, enzyme inhibitors play a major defensive role in protecting plants against insect pests by the inhibition of proteolytic enzymes in the digestive juices of insect pests. Proteinase inhibitors present in seeds and tubers of plants have been found to be active against a wide range of insects and have deleterious effects on plant pests by reducing rates of growth, development and reproduction in the insect populations (Broadway and Duffey 1986; Hilder et al. 1993). Proteinase inhibitors are generally categorized into four families according to the class of protease that they inhibit: serine, cysteine, aspartyl, and metallo proteinase. Insects use one or a combination of serine, cysteine, and aspartic proteinases as major digestive enzymes. Inhibitors of these

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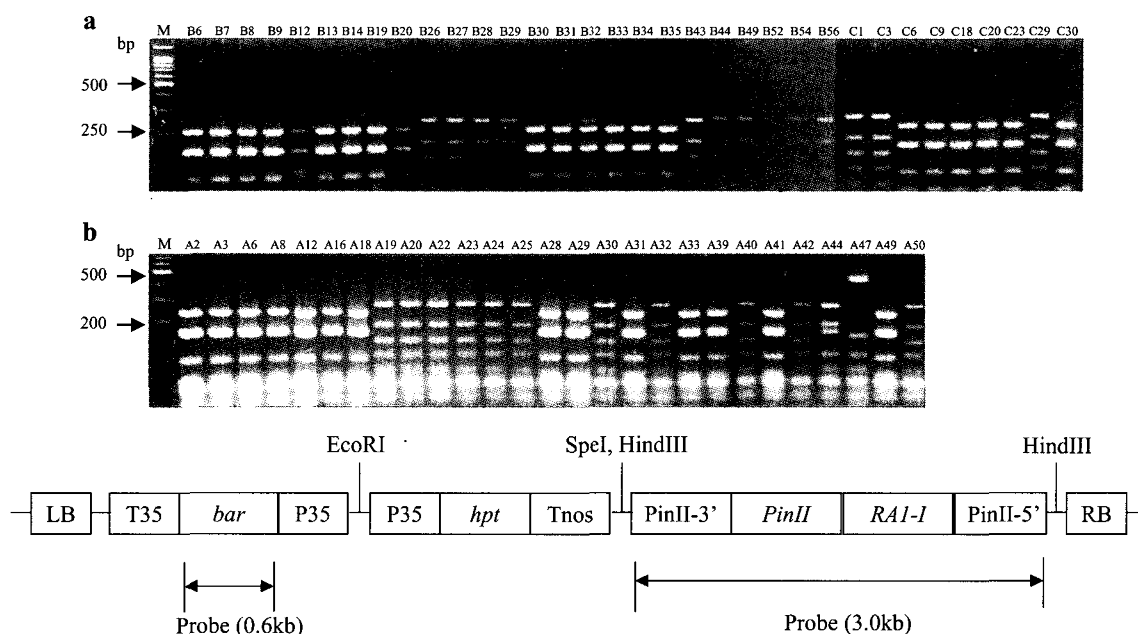
enzymes are synthesized by plants, and modulate the growth and development of pests by attenuating protein degradation. The Lepidoptera and Diptera orders possess mainly serine proteinases like trypsin and chymotrypsin. Serine proteinase inhibitors abundant in seeds and storage tissues have two active sites which inhibit trypsin and chymotrypsin. The CpT1 (trypsin/chymotrypsin inhibitor) isolated from cowpea was successfully transferred to tobacco and demonstrably enhanced resistance to insect attack (Hilder et al. 1987). The production of plants resistant to Lepidotera through the use of serine proteinase inhibitors of different origins has been subsequently reported. Tobacco and rice transgenic plants transformed with the serine PINII gene from potato showed enhanced resistance to *Menduca sexa*, *Chrysodeixis eriosoma*, *Seramia inferens*, and *Chilo suppressalis* (Johnson et al. 1989; McManus et al. 1994; Duan et al. 1996). Sweet potato trypsin inhibitors expressed in transgenic tobacco plants conferred resistance to *S. litura* (Yeh et al. 1997). Transgenic tobacco and peas expressing a proteinase inhibitor from Nicotiana alata showed increased *Helicoverpa armigera* resistance (Charity et al. 1999). Various plants such as tomato, lettuce, potato and rapeseed have also been genetically transformed (Gatehouse and Gatehouse 1998; Jouanin et al. 1998; Schuler et al. 1998). The high expression of toxins and proteinase inhibitors in transgenic plants may cause strong selective pressure on insect populations. In recent years, the constitutive expression of Bt genes in transgenic plants has caused the development of insect resistance to Bt toxins in the field (Shelton et al. 1993; Tabashnik 1994). Several cases of insect resistance to plant proteinase inhibitors have been reported (Broadway 1995; Giri et al. 1998; Jongsma and Bolter 1997; De Leo et al. 1998; Winterer and Bergelson 2001; De Leo and Gallerani 2002). Therefore, expression specific to tissue, growth stage and/or in response to insect feeding could reduce selective pressures and resultant insect resistance to toxins and proteinase inhibitors. In this study, the potato PINII gene with its own wound-inducible promoter and an herbicide resistance gene were introduced into commercial cabbage (*Brassica oleracea ssp. capitata*). The transgenic cabbage plants were analyzed and assayed against the larvae of diamondback moth and tobacco cutworm.

Materials and Methods

Construction of a plant expression vector pGR002

The 2.4kb EcoRI-HindIII fragment containing *p35::hpt::Tnos* of pIP101 (*p35::hpt::Tnos//PinII-5'::RA1-I::PinII::PinII-3'* in pUC12) was inserted into pCAMBIA 3300 to construct plasmid pGR001. The 3.0 kb HindIII-HindIII fragment containing *PinII-5'::RA1-I::PinII::PinII-3'* of pIP101 was transferred



Figuer 1. Construction map of the plant expression vector pGR002.
Bar: phosphinothricin acetyltransferase gene, *hpt*: hygromycin phosphotransferase, P35: CaMV 35S promoter, T35: CaMV 35S terminator, Tnos: transcription termination region of nopaline synthase gene, PinII-3': proteinase inhibitor II 3' region from potato, PinII: proteinase inhibitor II coding region, RA1-I: first intron of rice actin 1, PinII-5': proteinase inhibitor II 5' region.

to the HindIII site of pGR001 to produce the plant expression vector pGR002 (Explanation of abbreviations will be found in Figuer 1).

Cabbage transformation

Hypocotyls of cabbage, *B. oleracea* ssp. *capitata* UChi, grown *in vitro* were used for transformation. Cocultivation, selection and regeneration were carried out according to Lee et al. (2000).

Evaluation of herbicide tolerance and progeny analysis

An aqueous 0.6% solution of commercial Basta^R (PPT ammonium glufosinate content 18%) was applied to the leaves. Plants were scored for herbicide tolerance after 10-20 days. In order to obtain T_1 progeny seeds, transgenic plants were self-pollinated by bud pollination. Forty T_1 plants for each T_0 transgenic plant with 2-3 leaves were evaluated for Basta^R tolerance as above.

Southern hybridization analysis

Total genomic DNA was isolated from leaf tissue of T_0 transgenic plants according to Shure et al. (1983). The genomic DNA was digested with SpeI and separated in a 0.8% agarose gel. The gel was rinsed with distilled water and depurinated with 0.25N HCl for 5-10 min at room temperature. After washing with distilled water, the DNA was transferred to a Hybond N+ nylon membrane by capillary transfer under alkaline conditions. The membrane was rinsed briefly with 6XSSC, and prehybridized in a hybridization buffer (Church and Gilbert 1984) at 65℃ for 1 h. The PINII gene, labeled with [α-^{32}P]dCTP, was used as a probe (Figuer 1). After hybridization at 65℃ for 18 h, the membrane was washed for 15 min with 2XSSC, 0.1% SDS at room temperature, and then rinsed for 20 min with 1XSSC, 0.1% SDS at 65℃. The membrane was visualized using X-ray film.

Northern hybridization analysis

Total RNA was isolated from the leaf tissue of wild type, wounded wild type, and transgenic plants wounded by cutting according to Verwoerd et al. (1989). Leaf tissues were collected 8hr after wounding. Northern blot hybridization was carried out in accordance with the instruction manual from Hybond^{TM} membrane (Amersham Life Science).

Feeding Bioassays

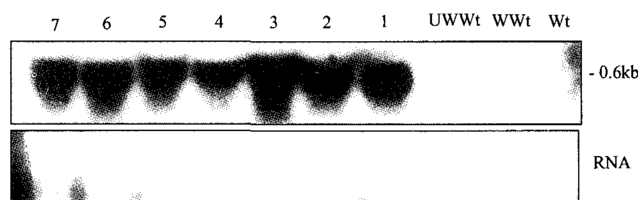
Second-third instar larvae of diamondback moth and tobacco cutworm were placed on detached leaves of T_1 transgenic cabbage (25-leaf stage, 28 days after transplanting) in three sets of petri dishes under 25℃, L:D= 16 : 8 photoperiod, and 60-70% relative humidity. Leaves were replaced with fresh ones every day. The weight increase of larvae (Weight Increase Index), pupation rate and adult emergence rate were used as evaluation parameters for determining the effects of transgenic cabbage on the development of diamondback moths. Weight Increase Index was calculated by dividing pupal weight by larval weight just after inoculation in diamondback moth, and dividing the weight of larvae fed on the plant three days after inoculation by larval weight just after inoculation in tobacco cutworm. Twenty larvae per treatment were used for this test, and each treatment was replicated three times. Weight increase values were compared among treatment using analysis of variance (ANOVA), and all data present means± SE. The analysis was performed using the statistics program SAS (SAS Institute Inc. 1985).

Results and Discussion

Production of transgenic cabbage plants and molecular analyses

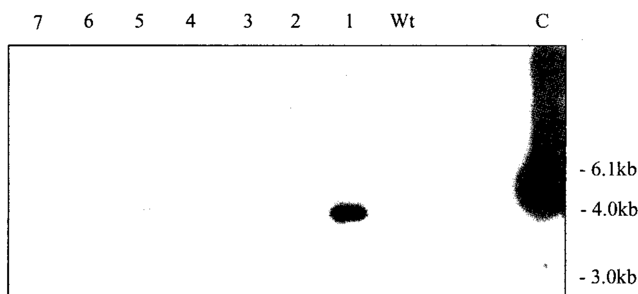
A total of fifteen transgenic plants were regenerated from the green compact callus derived from inoculated hypocotyls on medium supplemented with hygromycin as a selection antibiotic. All fifteen plants were derived from a single transformation experiment. Ten transgenic plants grew to maturity and set seeds by bud pollination. These transgenic cabbages showed herbicide resistance after application of Basta^R to leaves. The expression of the bar gene was confirmed by Northern hybridization analysis (Figuer 2).

The integration of the PINII gene into the cabbage genome

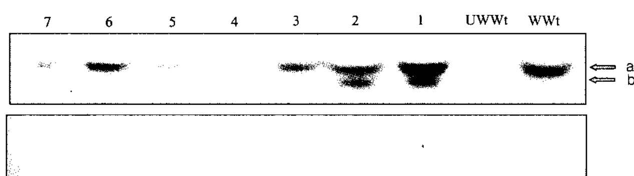


Figuer 2. Northern hybridization analysis of transgenic cabbages (T_0). Total RNA (20 μg) was hybridized with bar gene (probe 0.6 kb). Wt: wild-type plant, WWt: wounded wild-type plant, UWWt: unwounded wild-type plant. Lane 1-7: wounded transgenic plants, 1: TP3, 2: TP5, 3: TP6, 4: TP7, 5: TP8, 6: TP9, 7: TP10

was confirmed by performing Southern hybridization. One or two copies of the PINII transgenes were detected and, in addition, a putative homologous endogenous gene of cabbage was also found (Figuer 3). Expression of the proteinase inhibitor gene was examined in unwounded, wounded wild type and wounded transgenic plants. The transcripts of PINII (about 0.5 Kb) under a wound inducible promoter were produced in some transgenic plants (TP3, TP5 and TP10) wounded by cutting (Figuer 4). However, wound specific transcripts (about 0.6 Kb) of the endogenous proteinase inhibitor gene were also produced in wild type plant and transgenic plants but not in unwounded wild type plants (Figuer 4). This transcript is assumed to be from the endogenous proteinase inhibitor gene of cabbage already reported (Williams 1997). This gene encodes a serine proteinase inhibitor consisting of 214 amino acids. The difference in transcription of transgenes between transgenic plants might be explained by copy number, position effect and rearrangement of transgenes (Iyer et al. 2000). The herbicide Basta^R was applied to T_1 progeny of transgenic plants and T_1 plants resistant to herbicide were selected for bioassays.



Figuer 3. Southern hybridization analysis of transgenic cabbages (T_0). Genomic DNA was digested with SpeI and hybridized with PinII gene (probe 3.0 kb). C: pGR002 plasmid DNA, Wt: Wild-type plant, lane 1-7: transgenic plants, 1: TP1, 2: TP2, 3:TP3, 4: TP4, 5: TP5, 6: TP6, 7:TP10. Arrow: a putative homologous PINII gene of cabbage. The other hybridizing bands reflected the number of copies of integrated genes in transgenic plants.

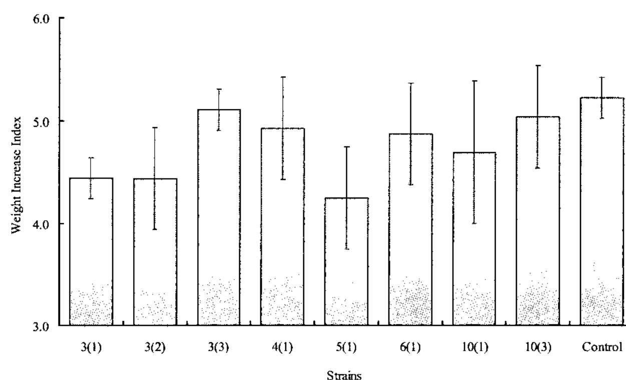


Figuer 4. Northern hybridization analysis of transgenic cabbages (T_0). Total RNA (20 μg) was hybridized with PinII gene. WWt: wounded wild-type plant, UWWt: unwounded wild-type plant. Lane 1-7: wounded transgenic plants. 1: TP3, 2: TP5, 3: TP6, 4: TP7, 5: TP8, 6: TP9, 7: TP10, a: endogenous proteinase inhibitor (0.6 kb) of cabbage, b: transgene proteinase inhibitor II (0.5 kb). TP3, TP5 and TP10 transgenic plants showed high expression of PINII transcript.

Bioassays against insect pests

Feeding bioassays were performed using the larvae of diamondback moth and tobacco cutworm with selected T_1 transgenic plants and a wild type plant as a control. These two Lepidopteran insects have serine proteinases similar to trypsin and chymotrypsin as digestion enzymes. The potato PINII protein is known to be a trypsin and chymotrypsin inhibitor (Koiwa et al. 1997). Duan et al. (1996) reported that transgenic rice transformed with potato PINII driven by its own promoter showed increased resistance to a major rice insect pest, pink stem borer.

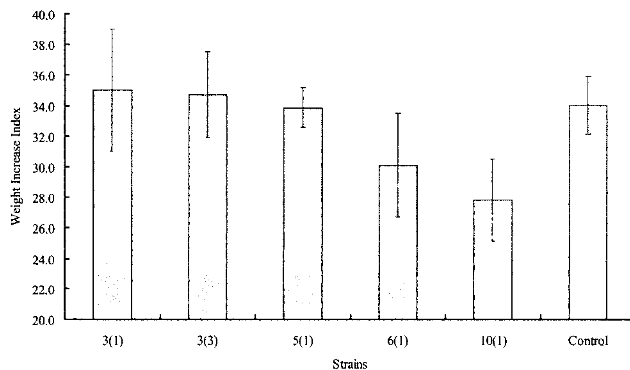
Second-third instar larvae of diamondback moths were placed on detached leaves of transgenic plants in petri dishes. The weight of larvae at inoculation and pupae grown on transgenic plants was compared (Figuer 5). The T1 transgenic plants showed various response. This difference could be explained by different expression of PINII transcript produced by segregation of transgenes in next generation. The weight of larvae and pupae grown on the transgenic plants 3(1), 3(3) and 5(1) was significantly lower when compared to control wild type plant. In addition, the pupation and emergence rate on transgenic plants 3(1), 3(3), 5(1), and 10(3) was significantly lower when compared to control wild type plants (Table 1). Transgenic plant 3(1) gave the lowest emergence rate (26.7%). Broadway (1995) reported that serine proteinase activity in the midguts of larval *P. xylostella* was moderately inhibited (40-50%) by the serine proteinase inhibitor of cabbage while pupation and adult emergence were not influenced. In this investigation, the pupation and emergence rate on some transgenic plants 3(1), 3(3), 5(1), and 10(3) decreased to 26.7%-36.7%. These plants were progeny of TP3, TP5 and TP10 displayed higher



Figuer 5. Comparative weight of larvae and pupae of Diamond backmoth, *Plutella xylostella* fed eight transgenic cabbage lines (T_1) under laboratory conditions. 3(1), 3(2), 3(3), 4(1), 5(1), 6(1), 10(1) and 10(3): progeny of TP3, TP4, TP5, TP6 and TP10 respectively.

Table 1. Comparative pupation and emergence rates of Diamond backmoth, *Plutella xylostella* fed eight transgenic cabbages (T_1) under laboratory conditions. 3(1), 3(2), 3(3), 4(1), 5(1), 6(1), 10(1) and 10(3): progeny of TP3, TP4, TP5, TP6 and TP10 respectively.

Strains	Pupation (%) ± SD	Emergence (%) ± SD
3(1)	40.0 ± 10.0	26.7 ± 5.8
3(2)	60.0 ± 10.0	60.0 ± 10.0
3(3)	36.7 ± 11.5	26.7 ± 11.5
4(1)	53.3 ± 11.5	46.7 ± 15.3
5(1)	43.3 ± 15.3	36.7 ± 11.5
6(1)	56.7 ± 11.5	53.3 ± 15.3
10(1)	60.0 ± 10.0	56.7 ± 5.8
10(3)	43.3 ± 5.8	36.7 ± 5.8
Control	76.7 ± 5.8	63.3 ± 5.8



Figuer 6. Increase in weight of tobacco cutworm, *Spodoptera litura* fed eight transgenic cabbages (T_1) under laboratory conditions. 3(1), 3(3), 5(1), 6(1), and 10(1): progeny of TP3, TP5, TP6, and TP10 respectively.

expression of PINII transcript. Therefore, it is presumed that this reduction was caused by expression of the introduced PINII gene.

Second-third instar larvae of tobacco cutworms were placed on detached leaves and the weight of larvae and pupa was measured every three days. Only 10(1) decreased significantly the weight of larvae and pupa grown on the transgenic plants when compared to wild type plants (Figuer 6). Tobacco cutworms grown on transgenic plant 3(1) showed the lowest pupation rate (50.0%). Larvae grown on transgenic plants 3(1), 6(1), and 10(1) had a lower emergence rate than on wild type plants (Table 2). It seems likely that expression of the potato PINII in transgenic cabbage was effective in the control of diamondback moths and tobacco cutworms. Moreover, the transgenic plants that caused a decrease in larval weight, pupation and emergence rate would be even more effective on larvae younger than 3rd instar, especially neonates. Yeh et el. (1997) reported

Table 2. Comparative pupation and emergence rates of tobacco cutworm, *Spodoptera litura* fed eight transgenic cabbages (T_1) under laboratory conditions. 3(1), 3(3), 5(1), 6(1), and 10(1): progeny of TP3, TP5, TP6, and TP10 respectively.

Strains	Pupation (%) ± SD	Emergence (%) ± SD
3(1)	50.0 ± 10.0	36.7 ± 5.8
3(3)	83.3 ± 5.8	50.0 ± 17.3
5(1)	76.7 ± 5.8	66.7 ± 11.5
6(1)	73.3 ± 15.3	30.0 ± 10.0
10(1)	83.3 ± 15.3	26.7 ± 11.5
Control	86.7 ± 11.5	70.0 ± 10.0

that growth of *S. litura* and *P. xylostella* on transgenic tobacco plants transformed with sweet potato trypsin inhibitor gene was severely retarded as compared to their growth on control plants. The percentage of larvae reaching adult stage was 13.2% in *S. litura* and 21.6% in *P. xylostella* (Yeh et al. 1997). In this investigation, the lowest emergence rate on transgenic cabbage was 30.0% in tobacco cutworms and 26.7% in diamondback moths. Therefore, expression of the PINII gene in transgenic cabbage had a significant influence on the emergence of adults. These results suggest that potato PINII controlled by a wound-inducible promoter in transgenic cabbage may be used to control insect pests through the reduction of insect progeny number .

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