

ISOLATION AND CHARACTERIZATION OF A SERINE PROTEINASE INHIBITOR cDNA FROM CABBAGE AND ITS ANTIBIOSIS IN TRANSGENIC TOBACCO PLANTS

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ABSTRACT

Plant proteinase inhibitors (PIs) are of special interest because of their role in plant defense against herbivorous insects. We isolated a cDNA clone for a serine PI from *Brassica oleracea*, cabbage (BoPI). A comparison of the putative coding sequence from the cabbage clone with soybean trypsin inhibitor identified conserved amino acids and peptide motifs. Furthermore, it seems to be a member of a 6-8 gene family in cabbage. The serine PI cDNA was subcloned into a plant expression vector under the control of the CaMV 35S promoter, and transgenic *Nicotiana tabacum* (tobacco) cv Xanthi were produced to test the ability of BoPI to enhance resistance against insects in a heterologous system. These plants were compared with transgenic plants containing different insect resistance transgenes (proteinase inhibitors and a *Bacillus thuringiensis cry1Ac*). The transgenic plants containing BoPI gene outperformed over other transgenic plants produced with different PI genes, and compared favorably with Bt cry1Ac transgenic plants in a bioassay with *Heliothis virescens*, tobacco budworm.

Key Words: *Brassica*, Herbivory, Insect Resistance, Proteinase Inhibitor, Transgenic Plants

Abbreviations: Bt, *Bacillus thuringiensis*; CEW, corn earworm (*Helicoverpa zea*); TBW, tobacco budworm (*Heliothis virescens*); YDK, Bt-susceptible TBW; YDH2, Bt-resistant TBW.

Nucleotide sequence appears in the Gene Bank, EMBL and DDBJ under the Accession No. U18995.

INTRODUCTION

Plant genes that protect against herbivory may be useful for heterologous expression into food and fiber crops (Boulter, 1989, 1993; Vogel et al., 1968). Proteinase inhibitors (PIs) are common in plants and have drawn attention as possible transgenes for insect defense in crops. PIs are of particular interest because they are generally the product of a single gene, and inhibit proteolytic enzymes of animal and fungal origin, but rarely plant origin, and therefore are thought to act as protective agents (Baldwin and Schultz, 1983; Brattsten, 1991; Green and Ryan, 1972; Hilder et al., 1993; Laskowski and Sealock, 1977). Several studies have demonstrated that PIs might provide adequate protection against a variety of economically important lepidopteran insects (Broadway and Duffey, 1986; Hoy and Shelton, 1987; Johnson et al., 1989; Lipke et al., 1954; Oppert et al., 1993; Sanchez-Serrano et al., 1987; Thomas et al., 1994, 1995; Xu, et al., 1996).

The production and accumulation of PIs in plants can be activated by a variety of mechanisms. Potato, tomato and poplar PIs have been shown to be wound inducible, both at the site of wounding and systemically (Bradshaw et al., 1990; Graham et al., 1986; Sanchez-Serrano et al., 1986). In contrast, the production of PIs in cabbage (*Brassica oleraceae*), especially trypsin and chymotrypsin inhibitors, are linked to plant development (Broadway and Missurelli, 1990). Low levels of PI activity in cabbage are produced in young foliage in seedlings (Broadway and Missurelli, 1990; Broadway and Colvin, 1992). When the plant reaches the 11-13 leaf stage, the level of PI activity gradually increases in young leaves, and reaches a maximal level of activity in the young foliage on mature plants. The production of PIs in cabbage is synchronized with the appearance of herbivorous insects in the field. Thus, the PIs are present when the resistance factors are most needed against these pests (Broadway and Missurelli, 1990; Broadway and Colvin, 1992). In addition, cabbage foliar extracts containing PIs have been shown to significantly reduced growth and development of larval Lepidoptera and plant pathogenic fungi (Broadway, 1995;

Broadway and Colvin, 1992; Lorito, et al., 1994).

As the result of the significant biological activity of cabbage PIs against herbivores and pathogens, we cloned a PI gene and expressed it in transgenic plants to better understand these molecules and to explore their effectiveness. We were interested in determining if expression of a cabbage PI in a heterologous system leads to an increased resistance to insect damage. This report includes the isolation and sequence characterization of a serine PI cDNA clone from cabbage (*Brassica oleracea* Proteinase Inhibitor, BoPI), and its heterologous expression in tobacco. In addition, we used a transgenic approach to compare BoPI to other insect resistance genes. We compared BoPI with *Bacillus thuringiensis* Cry1Ac (Bt) (Stewart et al., 1996a), and three proteinase inhibitors from the lepidopteran *Manduca sexta* (Kanost et al., 1989). Previous work demonstrated that Bt Cry1Ac is very effective in controlling lepidopteran larvae such as *Heliothis virescens* (tobacco budworm) and *Helicoverpa zea* (corn earworm). The *M. sexta* PIs had affinity for trypsin (AT), chymotrypsin (AC) and anti-elastase (AE) (Kanost et al., 1989), and these PIs are effective in controlling thrips (Thomas et al., 1994) and whitefly (Thomas et al., 1995), but were untested against Lepidoptera.

MATERIALS AND METHODS

cDNA cloning, Sequencing and Analysis

A cabbage (*Brassica oleracea* var. *capitata* L cv. Superpack) cDNA library was constructed in Lambda Zap II with mRNA purified from the young leaves of mature cabbage plants (Napoli et al., 1990), shown previously to be a rich source of PI protein (Broadway and Missurelli, 1990). This library was screened with antibodies produced in rabbits against affinity-purified cabbage PI (Broadway, 1993). Several positive clones were identified. An 809 bp clone was plaque-purified and the insert sequenced using terminator cycle sequencing and an Applied Biosystems (Foster City, CA) fluorescence sequencer. The 809 bp *Brassica oleraceae* proteinase inhibitor (*bopi*) coding region was isolated as a

EcoRI-XhoI restriction fragment then subcloned into pBluescript II SK (Stratagene, USA).

The *bopi* cDNA was isolated from pBluescript II SK by digestion with *XbaI* and *XhoI* to expose cohesive overhangs required for ligation into an intermediate cloning vector, pBJ40. The intermediate vector is a 14 kB plasmid that confers spectinomycin resistance in bacterial cells. The polylinker in pBJ40 is flanked by the cauliflower mosaic virus promoter (CaMV) 35S and nopaline synthase gene (nos) 3' terminator. The cassette was isolated as a *SacI-EcoRI* restriction fragment from pBJ40, and this was inserted between corresponding sites in the polylinker of the plant expression vector pBIN19. The plasmid was renamed pBIN/BoPI (Fig. 1). The plant transformation vectors containing Bt (Stewart et al., 1996b) and the *M. sexta* PIs (Thomas et al., 1994) were of similar construction as BoPI, with the antibiosis transgenes under the control of the 35S promoter, and kanamycin as the plant selectable marker.

Transgenic Plants

Transgenic tobacco (*Nicotiana tabacum* cv Xanthi) plants were produced using published methods (Horsch et al. 1985). Primary transformants were grown in a growth chamber with $500 \mu\text{E m}^{-2} \text{s}^{-1}$ irradiance and 12-h photoperiods to maintain plants for the insect bioassays. Plants were watered three times a week and fertilized as needed. Plant DNA was isolated according to published methods (Doyle and Doyle, 1987, Stewart and Via, 1993). Plant DNA samples were quantified by fluorometric spectrophotometry (Hoefer DyNA Quant 200, Hoefer Pharmacia Biotech Inc, USA). PCR was used to assess transgenic state of the plants (Table 1). Standard PCR was performed by 40 cycles of 94°C, 55°C, and 72°C. Ethidium bromide stained agarose gel electrophoresis was used to visualize PCR products. Transgene expression or activity was estimated using protein blot analysis for Bt (Stewart et al. 1996a) or enzyme assays for PIs.

Enzyme Assays

The initial enzyme assays performed on bulk numbers of transgenic plants were performed as follows. The determination of trypsin, chymotrypsin or elastase inhibition activity in transgenic plants was determined by a modification of the methods described by Geiger and Fritz (1983). Approximately 0.5 gm of fresh leaf tissue was homogenized in 5 ml of cold extraction buffer (25 mM NaH₂PO₄ pH 7.0, 10 mM EDTA free acid, 1% Sarkosyl, 1% Triton-X 100). Each homogenate was poured into a sterile, 50 ml conical centrifuge tube and diluted with an additional 20 ml of extraction buffer. A portion of each extract was used in a Bradford analysis (BioRad, USA) to determine the total soluble protein concentration. Each sample was assayed in quadruplicate by combining 100 μg of total plant protein and the appropriate amount of extraction buffer to bring each reaction volume to 800 μl in a 1 ml quartz cuvette. After briefly mixing the reaction mixtures, 200 μl of BAPNA (1.74 mg ml^{-1}) (*N* α -benzoyl-DL-arginine p-nitroanilide, Sigma) was mixed into each sample. A standard containing 2.5 mg/ml bovine trypsin (or chymotrypsin or elastase, as appropriate) and a blank solution lacking the BAPNA substrate were prepared to accompany each set of plant samples. All the cuvettes were incubated at 25°C for 10 min after the addition of all reagents. The absorbance of each sample was recorded by spectrophotometry at 410 nm using the substrate blank and the standard as a reference. The resulting spectrophotometric data were used to calculate the percent inhibition of each PI/plant sample.

Subsequent enzyme assays from an improved procedure (Menges et al. 1997, Thompson et al. 2000) were performed on leaves extracts from individual plants (Fig 4). Approximately 2 gm of fresh leaf tissue was macerated in 2 ml of homogenization buffer (100 mM Tris-HCl pH 7.5, 0.1 M CaCl₂) using a semi-automated plant tissue homogenizer (Bioreba Inc, USA). The homogenate was transferred to a clean 1.5 ml centrifuge tube and clarified by centrifugation at 15,000 rpm, 4°C for 10 minutes. Total protein of each plant extract was assessed by BCA assay (Pierce, USA). For inhibition assays, 10 mg/ml working stocks of trypsin and trypsin-

Table 1. Primer sequences and predicted DNA product sizes of transgenes.

Gene & Primer(s)	Product (bp)	Primer (bp)	Primer Sequence
BoPi Forward Reverse	455	21mer 18mer	GGCAGTTACTACGTTCTCCCC CGATAGGGGTAGCGAATG
<i>M. sexta</i> Pi's Forward Reverse	770	20mer 21mer	ACGACCAAT TTACAGCCCAG GTTGTACAAACGCTTCCCTCAGC
Cry1Ac Forward Reverse	560	20mer 20mer	ATTTGGGGAATCTTTGGTCC ACAGTACGGATTGGGTAGCG

* bp, Base pairs

Table 2. Transformation efficiency of tobacco plants subjected to *Agrobacterium* mediated gene transfer.

Transgene	Start ^a	Callus ^b	Shoots ^c	Rooted ^d	No of lines ^e
sBt	300	137 (46%)	50	38 (76%)	21
<i>M. sexta</i> AE	300	126 (42%)	50	28 (56%)	18
<i>M. sexta</i> AC	300	---	50	---	---
<i>M. sexta</i> AT	300	95 (32%)	50	---	---
			50		14

^a Number of leaf disks co-cultivated in *Agrobacterium*. ^b Number of leaf disks that produced callus during antibiotic selection. ^c Total number of shoots excised from callus. ^d Number of rooted shoots, each representing unique transgenic events.

chymotrypsin protease inhibitor (Cat No. T-7409 and T-9777 respectively, Sigma Chemical Co., USA) were prepared by dissolving each compound in digestion buffer (10 mM Tris-HCl, pH 7.8, containing 0.1 mM sodium azide).

Protease activity was determined using a fluorescence-based assay (EnzChek BODIPY Fluorescence Kit, Cat No. E-6638, Molecular Probes, USA) in a 96-well format. Assays were carried out in duplicate and blanks were used to account for any background. A standard curve was generated to determine the activity of purified protease, and to determine the effect of varying levels of purified inhibitor on a known concentration of protease. To detect protease-inhibitor activity in the plant extracts, 25 µg of soluble protein was loaded into the well of a flat bottom, black 96-well plate (Costar Brand, USA). Enough trypsin was added to each sample so that a final concentration of 2.5 µg/ml would be achieved. Enough digestion buffer was added to each sample to bring the volume to 100 µl. Finally, 100 µl of the BODIPY casein working solution was added. The microplate was incubated for 1 hour at room temperature, protected from light.

Fluorescence was measured with a filter fluorometer (excitation 485 nm, emission 535 nm) on a SpectraFluor Plus microplate multi-detection plate reader controlled by X-Flor acquisition utility software (TECAN Instruments, USA).

Insect Bioassays of Transgenic Plants

The bioassay arenas were constructed using a 100-mm x 10-mm polystyrene petri dish. A single 10-cm diameter filter paper was placed in the bottom of the dish then moistened with 1-2 ml of deionized water. Leaf disks were cut from mature tobacco leaves using a 4-cm diameter leaf borer with a beveled cutting edge that was fashioned from a length of copper pipe. Two leaf disks from each plant were placed on the filter paper, then the arena was covered using the lid of the petri dish. Three replicates for each plant line were prepared.

The lepidopteran species used in this experiment were the *Bacillus thuringensis* (Bt) susceptible tobacco budworm (*Heliothis*

virescens (strain YDK)), the Bt-resistant tobacco budworm (strain YHD2) (Gould et al., 1995), and corn earworm (*Helicoverpa zea*). The eggs of each insect strain were incubated in 30-ml transparent plastic cups with unwaxed cardboard lids at 20°C until they hatched. Three newly hatched larvae were removed from the plastic cups and applied to each leaf disk in a bioassay arena using a fine hair paint brush, and the lid placed back on the bioassay arena. The bioassays were maintained on a stainless steel wire shelf that allowed even air circulation, and the bioassays were conducted for five days, with periodic watering of the disks to maintain the health of the leaf disks. The treatments were maintained at 25°C and constantly illuminated with fluorescent lighting throughout the experiment. Data were collected at the conclusion of each trial measuring the antibiosis parameters: percent mortality, body length of surviving insects, and defoliation of the leaf disks. The data were analyzed using SAS. A two-way analysis of variance (ANOVA) was performed using the categorical variables transgenic plant type and insect species to determine any significant differences with respect to the antibiosis parameters. Multiple comparisons using Tukey's studentized range test were performed on all main effect means.

RESULTS

BoPI 1-2 codes for a Serine Proteinase Inhibitor

The clone (*bopi* 1-2) was 809 bp and contained the complete coding sequence as well as the complete 3' noncoding sequences (data not shown). The clone contained an open reading frame (orf) of 642 bp starting at nucleotide 2. The orf contained a typical hydrophobic signal sequence at residues 1-21. The amino acid residues at positions 22-41 corresponded to the amino-terminal sequence determined for the most abundant mature PI (Fig. 3). The predicted mature peptide had a calculated molecular weight of 21 kDa and a calculated pI of 4.94. These values fall within the range reported previously for cabbage PIs (Broadway, 1993).

The peptide encoded by *bopi* 1-2 was predicted to be a member of the soybean Kunitz class of trypsin inhibitors. It also showed significant similarity to the α -amylase/subtilisin inhibitors of cereals. The predicted peptide had a 30% identity to soybean trypsin inhibitor-3 (Jofuku et al., 1989). The predicted BoPI peptide contained the sequence VLDTDGDIIFDGSYYVL at residues 24-40, which matched the signature pattern $\{LIVD\}\text{-x-D-x}\{EDNTY\}\{DG\}\{RKHDENQ\}\text{-x}\{LIVM\}\text{-x(5)-Y-x}\{LIVM\}$ found in the amino-terminal section of Kunitz inhibitor family members (Bairoch, 1991). The arginine residue at position 63 corresponding to active site arginine of soybean trypsin inhibitor (Sweet et al., 1974) and four cysteines that could facilitate intrachain disulfide bonds (Laskowski and Kato, 1980) were conserved in the cabbage sequence. A drought-induced protein related to the Kunitz trypsin inhibitor family was cloned in *B. napus* (Downing et al., 1992). The conserved amino-terminal motif (residues 23-39) and the first cysteine pair is present in the *B. napus* sequence but not the active site arginine or the second cysteine pair (Fig. 3) (Downing et al., 1992). Overall, the predicted BoPI peptide had slightly less identity to the *B. napus* peptide (46/214 identical residues) compared to the soy peptide (56/214).

Cabbage pi Gene Family

Genomic Southern blotting (Fig. 2) and analysis of genomic clones (data not shown) suggested that BoPI was present as a member of a small gene family. This is similar to soybean (Jofuku and Goldberg, 1989) and potato (Ishikawa et al., 1994) proteinase inhibitors and winged bean chymotrypsin inhibitor (Habu et al., 1992) gene families. A cabbage genomic library in Lambda Fix II (Stratagene) was constructed and screened with a cabbage *bopi* 1-2 cDNA. Six positive clones were purified and shown to fall into 2 distinct classes by restriction digestion analysis. PCR amplification of the *bopi* coding sequences in the genomic clones suggested that these cabbage *bopi* genes are intronless (not shown). Cabbage genomic DNA was digested with enzymes that do not have sites in the *bopi* 1-2 cDNA clone, and analyzed by Southern blotting. Several bands (6-8) of

varying intensity were seen in each digest (Fig 2).

Transgenic Plants

We recovered multiple events of independently transformed lines containing each gene of interest from 300 leaf disks taken from an inbred cultivar of tobacco (Xanthi) transformed with *Agrobacterium* (Fig. 5) (Table 2). All plants were morphologically normal and fertile. Southern blot analysis demonstrated that all transgenes were integrated with 1-4 copies contained in plants (data not shown).

Transgene Expression

Bt transgenic plants had similar expression levels compared with earlier work with transgenic canola (Stewart et al., 1996b). There was a range of low (0.005%) to high (0.1%) expression levels in transgenic plants. Likewise, enzyme analysis of PI transgenics showed a range of expression as determined by the hydrolysis of BAPNA by the appropriate digestive enzyme. For example, BoPI transgenic plants (n=11) inhibited the release of *p*-nitroaniline from 6.31% to 27.61% compared to wild-type plants. There were no significant differences in proteinase inhibition among PIs at the P=0.05 level. In the subsequent enzyme assays to more accurately assess individual plant line differences using an improved method (Menges et al. 1997, Thompson et al. 2000), the same relative patterns of transgenic expressed PI to endogenous PI were similar (Fig 4). However, there was great variation among lines of all transgenic plants' expression of the transgenes, as well as endogenous PI (Fig. 4).

Insect Bioassays and Plant Performance

Insect assays and protein assays were performed on the same plants. In general, the growth and defoliation by the insects on wild-type (WT) leaf disks was higher than transgenic leaf disks for all three insects (P < 0.05) (Fig. 6). Likewise, larval corn earworm (CEW) survived significantly better on WT leaves than on any of the transgenics (P<0.05). All CEW that fed on transgenic

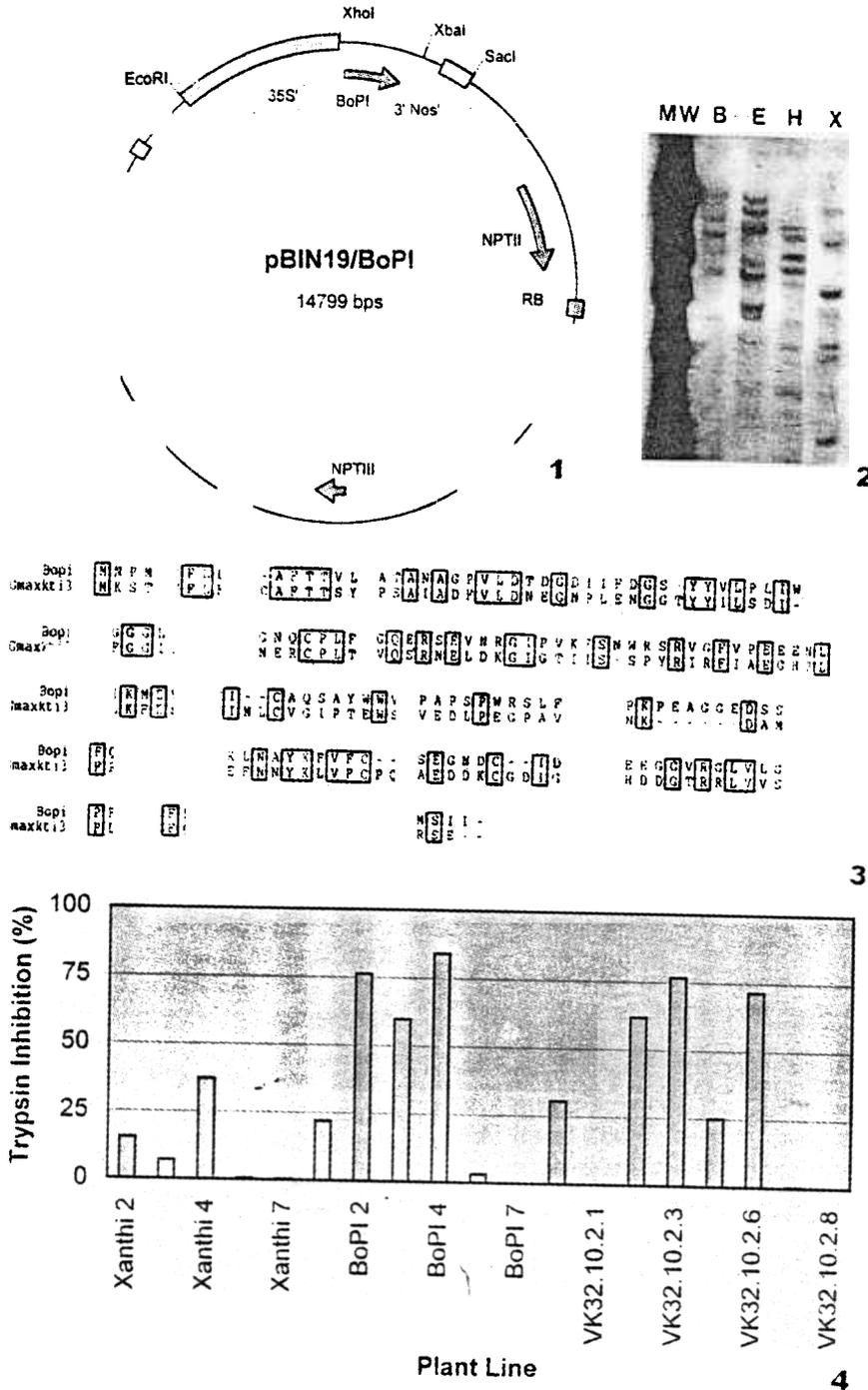


Figure 1. Plasmid map of pBIN/BoPI. Figure 2. Cabbage genomic Southern. Digested cabbage genomic DNA was fractionated by gel electrophoresis, transferred to a nylon membrane (Sambrook et al., 1989) and hybridized to digoxigenin-labeled cabbage pin 1-2 according to manufacturer's instructions (Boehringer-Mannheim, Indianapolis). The final wash conditions were 0.1x SSC at 55 °C. Hybridizing bands were visualized with Lumi-Phos 530 after exposure to film for 2 hours. Lanes: MW, digoxigenin-UTP-tailed HindIII digest of lambda DNA (bands at 23, 9.4, 6.5, 4.4, 2.3, 2.0 and 0.56 kb); B, BamHI; E, EcoRI; H, HindIII; X, XbaI. Figure 3. Comparison of the amino acid sequence derived from the bopi cDNA clone from cabbage (*B. oleracea*) with soybean Kunitz-type trypsin inhibitor 3 (*G. max*) (Jofuku et al., 1989) and the drought-induced BnD22 from *B. napus* (Downing et al., 1992). The amino-terminal peptide sequence of the *B. oleracea* PI, residues 22-41, was determined at Cornell Biotechnology Facility. The active site arginines in the *B. oleracea* and *G. max* PIs are shown with a closed circle, conserved cysteine residues are shown with triangles and the cleavage site to produce the mature cabbage peptide is shown with an arrow. The predicted PI peptides contained sequences starting at residues 24, 27, and 23 for *B. oleracea*, *G. max*, and *B. napus* respectively which matched the signature pattern ((LIVM)-x-D-x-{EDNTY}-[DG]-[RKHDENQ]-x-[LIVM]-x(5)-Y-x-[LIVM]) found in the amino-terminal section of Kunitz inhibitor family members (Bairoch, 1991). Figure 4. Enzyme (trypsin or chymotrypsin) assays performed on wildtype tobacco (Xanthi), BoPI and *Manduca sexta* anti-chymotrypsin (VK32) individual events (transgenic T1 progeny).

tobacco expressing the Bt endotoxin were killed. However, there was more survivorship of CEW when fed on tobacco expressing PI proteins compared with Bt (Fig. 6). Survivorship of CEW on plants expressing PI proteins (BoPI, *M. sexta*-AT, AC and AE) was similar. Survivorship of CEW on Bt transgenic plants was significantly different than CEW on PI transgenic plants ($P < 0.05$).

A low, but comparable level of survivorship was observed in YHD2 insects on Bt and BoPI plants. However, survivorship was high for YHD2 on plants expressing the *M. sexta* derived PIs. Moreover, the survivorship of YHD2 that fed on plants expressing the *M. sexta* PIs was similar to that of YHD2 on wild-type plants. The mortality of YHD2 observed in Bt and BoPI transgenics was significantly different than the survivorship of YHD2 observed in the *M. sexta* derived PI transgenics ($P < 0.05$). This trend was also observed in the YDK strain of *H. virescens*. Both Bt and BoPI transgenics were associated with similar levels of mortality of YDK insects. However, survivorship of YDK insects on any *M. sexta* transgenic plant showed survivability similar to YDK on WT plants. The survival of YDK on Bt and BoPI transgenic plants was similar, and the values were significantly lower than the survivorship of YDK on any of the *M. sexta* derived PI transgenics ($P < 0.05$).

As anticipated, the average defoliation by CEW, YDK and YHD2 on wild-type plants was significantly higher ($P < 0.05$) than the defoliation observed on the transgenic plants (Fig. 6). The average defoliation by CEW in all transgenics was similar, and no significant differences in defoliation by CEW were observed among transgene type ($P < 0.05$). However, defoliation by CEW was slightly higher among plants expressing proteinase inhibitors than in Bt plants. Likewise, the average defoliation by YHD2 in all transgenics was similar, and no significant differences were observed among transgene type. Defoliation by YHD2 was slightly higher among plants expressing proteinase inhibitors than in Bt plants. Defoliation by YDK in plants expressing the *M. sexta* AT and AE proteinase inhibitor proteins had defoliation levels similar to non-transgenic plants. There were no differences in the average defoliation by YDK on Bt, BoPI and the *M. sexta* AC transgenics.

The average length of CEW on Bt plants was smaller than CEW on any plant expressing a proteinase inhibitor protein (Fig. 6). The average length of *H. virescens* (YDK and YHD2) on Bt and BoPI plants were similar. These insects were smaller than *H. virescens* on the plants expressing the *M. sexta* proteinase inhibitors. However, the means observed between Bt and BoPI, versus the *M. sexta* PIs were not significantly different ($P < 0.05$).

DISCUSSION

The growth and development of caterpillars are significantly reduced when on plants transformed with Bt or PI (Hoffmann et al., 1992; Hua et al., 1993; MacIntosh et al., 1990a; Santos et al., 1997). These studies established that Bt is more effective than PIs in controlling lepidopteran insects. The findings in the present study expanded this observation to an array of serine proteinase inhibitor genes, including anti-trypsin, anti-elastase and anti-chymotrypsin PIs, and compared their effectiveness against Bt-susceptible and Bt-resistant caterpillars. As expected, tobacco transformed with Bt *Cry1Ac* endotoxin was more effective in killing insects than any of the PIs examined in this study, with the exception of BoPI on TBW survivorship in the bioassay. This profound difference between Bt and the PIs can be attributed to mode of action. The effect of Bt ingestion is immediate and more lethal than that of the PIs. Ingestion of the Bt endotoxin induces pore formation in the cells in the insect midgut, causing death within hours. In contrast, ingestion of PIs will ultimately lead to a decline in the feeding behavior of the insects, resulting in a decrease in growth, causing death in several days.

The cabbage-derived PI (BoPI) used in this study shows promise as a useful natural insecticide against certain lepidopteran insects. BoPI effectively reduced survivorship of the Bt-resistant (YHD2) and Bt-susceptible (YDK) strains of tobacco budworm, exhibiting levels similar to plants producing the *Cry1Ac* toxin. However, BoPI plants were not as effective at reducing survivorship of corn earworm (CEW). This finding is consistent with a previous study on CEW that

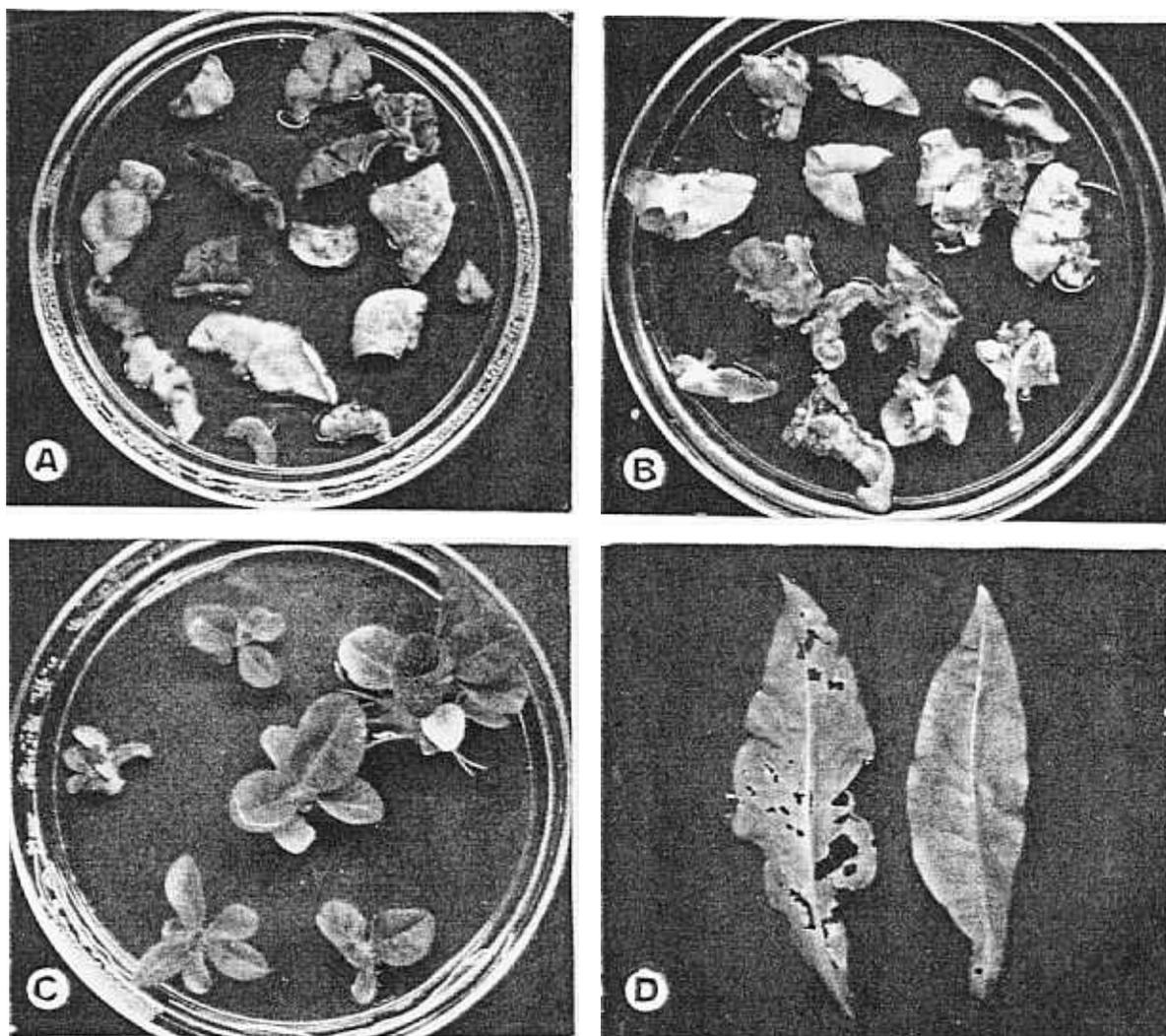


Figure 5. Development of tobacco transgenic plants; A-Leaf explants for cocultivation; B-Shoot bud development from cocultivated explants; C-Transgenic plants growing on medium and D-Comparison of transgenic leaf performance with wild leaf to insect bioassay.

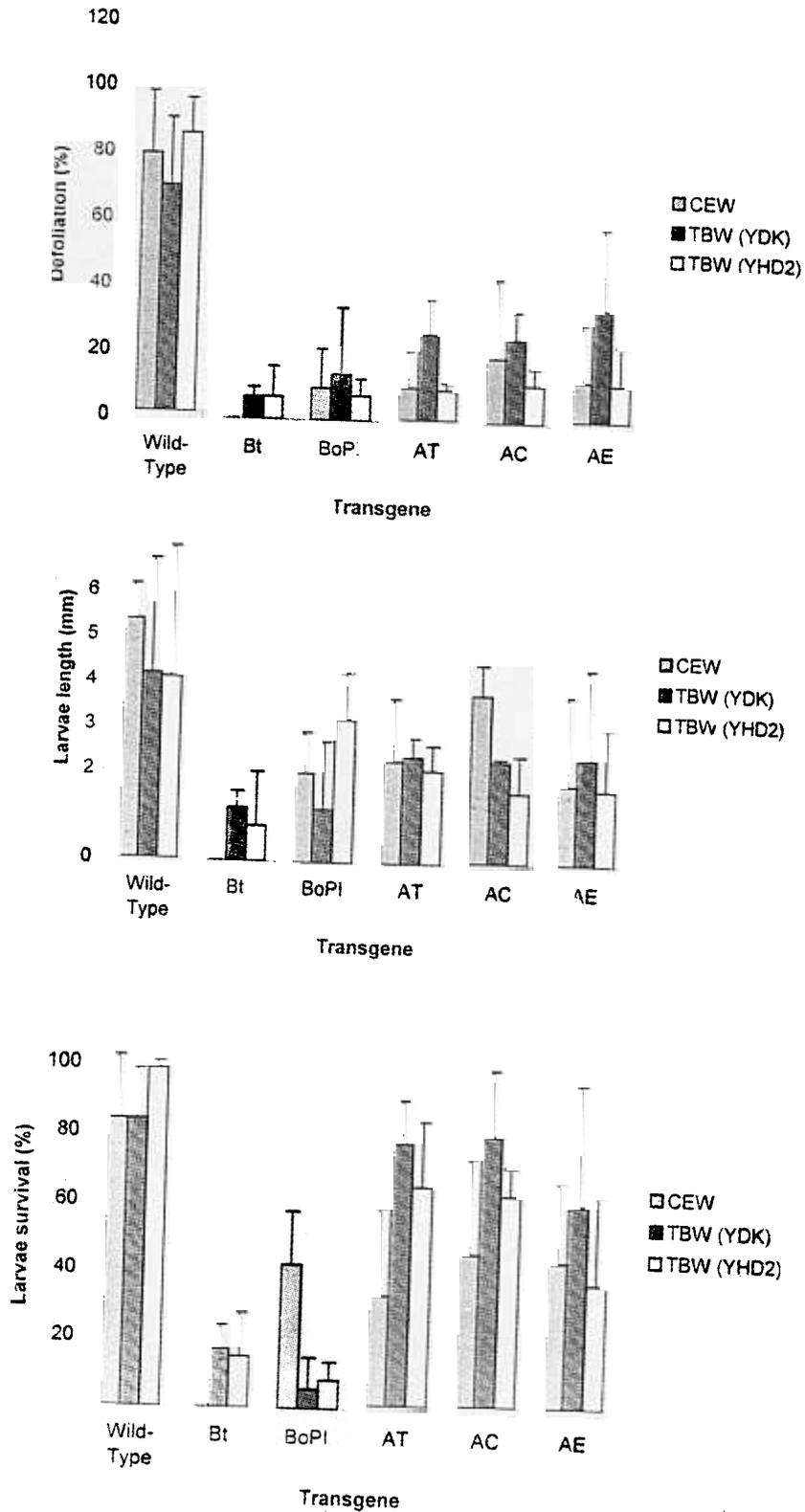


Figure 6. Performance of corn earworm (CEW), tobacco budworm Bt-susceptible (YDK), and tobacco budworm Bt-resistant (YHD2) on wild-type, *Bt*, *Brassica oleraceae* PI (BoPI), *Manduca sexta* anti-trypsin (AT), anti-chymotrypsin (AC) and anti-elastase (AE) PI transgenic tobacco leaf discs.

incorporated cabbage proteinase inhibitors into artificial diets (Broadway, 1995, 1996). Broadway investigated the potential of herbivorous insects to become resistant to plant proteinase inhibitors, under the hypothesis that long-term exposure to certain proteinase inhibitors would reduce the toxic effects of subsequent exposure to those toxins. For instance, diamondback moth (*Plutella xylostella*), imported cabbageworm (*Pieris rapae*), and cabbage loopers (*Trichoplusia ni*) are all cabbage specialists, and possess adaptive defenses to cabbage phytochemicals. Hypothetically, they should be more resistant to PIs in cabbage. In contrast, generalists like CEW, an insect that does not feed on cabbage, should be more susceptible to cabbage PIs. *In vitro* inhibition studies demonstrated that the trypsins from imported cabbageworm were not susceptible to inhibition by cabbage PI, while the trypsins from CEW were significantly inhibited by cabbage PI (supporting the hypothesis). However, feeding studies using artificial diet demonstrated that growth and development for both species was not effected by ingestion of cabbage PI (Broadway, 1995). Similar results were found when the insects were feeding on plant tissue containing trypsin inhibitors (Broadway and Colvin, 1992; Broadway 1995). In contrast, the cabbage looper was susceptible to cabbage PI as demonstrated by *in vitro* inhibition studies and ingestion studies. That study demonstrated that some lepidopterans have the ability to adapt to certain PIs by secreting a suite of enzymes that are not susceptible to those inhibitors (Broadway, 1996). In the present study, survivorship and defoliation levels by corn earworm on BoPI plants, were similar to those observed on plants expressing the *Manduca sexta* derived serine PIs, confirming that CEW is not susceptible to cabbage PI.

Another interesting observation of the present study was the heightened sensitivity of CEW to *Bt*. It is not clear why CEW was completely controlled in this study by Cry1Ac. Since we had a range of *Bt* expression, we expected some larval survival. Previous investigations have reported that CEW was less sensitive to the Cry1Ac delta-endotoxin than the observations in the present study (Luttrell et al., 1999; MacIntosh et al., 1990b; Sims et al., 1996). Stone and Sims (1993)

reported significant differences in the susceptibility to *Bt* (Cry1Ac) among field strains of TBW and CEW. Of the two species, CEW had a higher tolerance for Cry1Ac. It was also unusual to observe that there were no differences in performance between TDK and YDH2 when allowed to feed on *Bt*-transgenic plants. While we have no explanations for these apparently aberrant results, the BoPI transgenic plants performed comparably to *Bt* in TBW control.

While BoPI does have some merit as a lepidopteran control agent, it will likely not perform well stacked with *Bt* in transgenic plants. As Santos et al. (1997) point out, PIs may inhibit proteinases needed to activate *Bt* protoxin into the insecticidal form. In BoPI x *Bt* hybrids, we have observed a significant decrease in antibiosis (data not shown). Nonetheless, BoPI might prove to be an effective PI for lepidopteran control in transgenic plants when *Bt* is not desirable. It might also be useful when a plant-derived transgene is desired.

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