Expression of Soybean Proteinase Inhibitor in Sweetpotato

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Transformation of sweetpotato (*Ipomoea batatas*) with serine-type proteinase inhibitors (PIs) offer a new strategy for developing host resistance against sweetpotato weevil (*Cylas* spp.), the major threat to sweetpotato worldwide. Foreign genes encoding PIs have been introduced into tobacco, tomato, strawberry, potato, and many other crops for control of a range of insect pests. These proteins bind to proteinases in the midgut of the insect thus interfering with the insect's metabolism. Transgenic tobacco plants expressing recombined serine PIs have demonstrated enhanced resistance to Heliothis *virescens* (Hilder et al., 1987), *Manduca sexta* (Johnson et al., 1989), and *Spodoptera litura* (Yeh et al., 1997).

The predominance of serine-type proteases in the midgut of three *Cylas* spp. was confirmed by inhibition assays. Serine-type inhibitors, regardless of the species or developmental stage of the insect, inhibited the proteinases detected (Rety et al., 1998). The inhibitory spectrum of soybean (*Glycine max*) trypsin inhibitor (SBTI) against the midgut proteases was apparently larger than that of endogenous inhibitors in sweetpotato. It was apparently stable in the presence of insect insensitive proteinases, suggesting the potential usefulness of this inhibitor in controlling weevil larvae and adults (Zhang et al., 1997).

Newell et al. (1995) developed the first transgenic lines of PI-expressing sweetpotato with a cDNA sequence encoding a cowpea (*Vigna unguiculata*) trypsin inhibitor (CpTI). Feeding tests with the West Indian sweetpotato weevil (*Euscepes postfasciatus*) were carried out recently at CIP by performing a no-choice test in a screenhouse-based bioassay. Two transformed clones were found moderately resistant in consecutive tests over 2 years, suggesting the actual usefulness of the trypsin inhibitor in weevil control.

This paper describes the Agrobacterium-mediated transformation of sweetpotato with a soybean kunitz trypsin inhibitor (SKTI-4) cDNA clone and the analysis of recombinant SBTI produced in sweetpotato cells against trypsin and sweetpotato leaf proteinases.

Materials and Methods

Sweetpotato transformation with SKTI-4 gene

Complete leaves with petioles of in vitro plants of sweetpotato cv. Jewel, PI-318846-3, and Jonathan, and leaf segments of cv. Maria Angola were used as explants. Explants were inoculated and co-cultivated with *Agrobacterium tumefaciens* LBA4404 carrying plasmid pKTI-4. The T-DNA region of this plasmid harbors the SKTI-4 gene, the b-glucuronidase (GUS) reporter gene, and the NPTII marker gene (Figure 1).

The first three explants went through two successive transfers: First to F15 medium (Murashige-Skoog (MS) salts, 0.2 mg/L 2, 4-D; 0.2 mg/L zeatin; 50 ppm kanamycin-sulfate; 200 ppm cefotaxime; 30g/L sucrose; and 3.6 g/L phytagel) for 3 d; and second to F9 medium (same as F15 but without 2, 4-D). All calli formed were analyzed for GUS activity, and positive calli were transferred to G24D medium (basal medium supplemented with 0.2 mg/L 2,4-D

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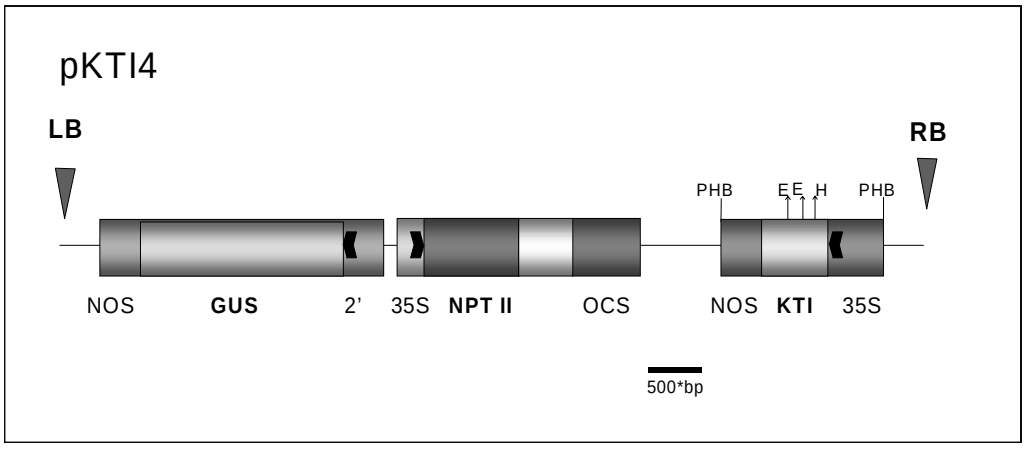

Figure 1. Diagram of T-DNA region of plasmid pKTI-4. Coding regions for ß-glucuronidase (GUS), neomycin phosphotransferase II (NPTII), and Kunitz trypsin inhibitor (KTI) genes are transcribed between TR 2' (2') or Cauliflower mosaic virus 35S (35S) promoter sequeneces and nopaline synthase (NOS) or octopine synthase (OCS) terminator sequences. The direction of transcription is shown by the arrowhead. Relevant restriction enzyme sites are indicated: Pac I (P), Hind III (H), Bam HI (B), Eco RI (E). Left border (LB), right border (RB), base pairs (bp).

and 0.05 mg/L gibberellic acid) for multiplication. After 2 mo they were transferred to auxin-free media for regeneration.

Leaf segments of Maria Angola, following inoculation and co-culture, were cultivated in medium G24D for 2 mo for embryogenic calli induction. These calli were evaluated for GUS activity and positive calli were transferred to MS30-Vit medium for embryo development.

GUS test of SKTI-4 transgenic lines

GUS expression in transformed plants was evaluated by histochemical assay as described by Jefferson (1987). Leaves, roots, and stem segments were incubated in phosphate buffer pH 7 with X-gluc (5-bromo-4-chloro-3-indolyl-beta-D-glucuronic acid) substrate. Blue staining of the tissue denoted positive reaction.

Kanamycin resistance evaluation of SKTI-4 transgenic lines

Three petridishes with five leaf segments (each segment corresponding to a different leaf) were cultivated for each putative transgenic line. Included in each petridish were two leaf segments of a known transformed line as a positive control. In addition, 2-4 leaf segments of an untransformed plant of the same cultivar were included as a negative control. Medium used for calli induction was 303-64 (MS salts, 1 mg/L zeatin, 0.1 mg/L naftalen-acetic acid, 30 g/L sucrose, and 3.6 g/L phytagel) containing a high concentration (100 mg/L) of the antibiotic kanamycin sulfate. As a control for the effect of the physiological status of the plants, or the effect of the medium, the same experiment was repeated with each line without kanamycin, and also with the untransformed cultivars with the same concentration of kanamycin. Calli development was evaluated after 1 mo in culture.

Southern blot test of transgenic lines

The presence of SKTI-4 sequence was confirmed by Southern blot analysis. DNA was isolated from 4 g of frozen leaf tissue from in vitro plantlets (Dellaporta et al., 1983). Ten micrograms of DNA were digested with restriction enzyme BamHI (the SKTI-4 gene has BamHI restriction sites at left and right borders as shown in plasmid pKTI-4, Figure 1), and separated by electrophoresis in a 0.9% agarose gel. The gel was treated for 10 min with a solution

containing 0.25N HCl, twice for 15 min in 0.5N NaOH, 1.5M NaCl, and twice for 30 min in 1M tris pH 7.4, 20 mM NaOH and was blotted to a nylon filter (Amersham) by capillarity.

One hundred nanograms DNA of SKTI-4 gene isolated by restriction of the plasmid pKTI-2 was radiolabeled with ^{32}P (dATP) and used as a probe for filter hybridization. After hybridization, the membrane was washed twice, for 5 and 15 min in a buffer solution containing 2X SSC, 0.5% sodium dodecyl sulfate (SDS) at room temperature. The membrane was washed twice more for 30 min each in 0.1 X SSC and 0.5% SDS, once at 37°C and once at 65°C. Filters were exposed for autoradiography with an intensifying screen at -70°C for up to 2 d.

PCR analysis of transgenic lines

The SKTI-4 gene was targeted with specific primers and amplified by polymerase chain reaction (PCR) from DNA of in vitro transgenic plants. These 18 base pairs (bp) forward and reverse primers where specially synthesized to flank the 653 bp of the SKTI-4 gene according to the complete PUC-derived pKTI-2 plasmid's sequence provided by C. Newell of Pestax Ltd., UK (Table 1).

The PCR amplification condition was as follows:

1 cycle:	94°C for 1′
35 cycles:	94°C for 1′
	52°C for 2′
	72°C for 3′
1 cycle:	72°C for 7′

For the amplification of each transgenic line, 40 ng of DNA extracted from in vitro plants were used. One nanogram of plasmid pKTI-2 (PUC-derived plasmid harboring the SKTI-4 gene) was used as a positive control. DNA of untransformed plants (C-) and the amplification mixture without DNA (M) were used as negative controls.

Protease and protease inhibitor assays

The protease assay was adapted from Michaud et al. (1993). Thirty-five µL of leaf protein extracts from 8 transgenic lines or from an untransformed control of genotype PI-318846-3 (1 µg protein/µL extract) were incubated with 5 mL of 0.5mg/mL (w/v) trypsin dissolved in 50 mM tris, pH 8.0. The samples were incubated for 15 min at 37°C to allow protease inhibition, and 80 µL of 2% (w/v) azocasein dissolved in the same buffer was added as substrate to each sample. After a 180-min incubation, 300 µL of 10% (w/v) trichloroacetic acid (TCA) was added to the mixture, and residual azocasein was removed by centrifugation for 5 min at 14,000 rpm. The supernatant (350 µL) was then added to 400 µL of 1N NaOH, and the absorbance was measured at 440 nm using a Spectronic spectrophotometer (Milton Roy, Rochester, NY, USA). The absorbance of blanks, which consisted of complete mixtures without trypsin or plant extract submitted to the same treatment, was subtracted from each value. One unit of protease activity was defined as the amount of enzyme required to produce an absorbance change of 1.0 h^{-1} in a 1 cm-cuvette, under the conditions of the assay. The results obtained were compared with controls consisting of (1) trypsin without plant extract (maximum hydrolysis), and (2) trypsin plus extract from an untransformed plant.

Electrophoretic analyses

Expression of proteinase inhibitors in the leaf tissues was visualized by polyacryla-

Table 1. Primer's sequence for PCR reaction.

Primer name	Corresponding sequence	Sequence	Melting temperature (°C)
SKTI2.1 (forward)	SKTI leader sequence	ATGAAGAGCACCATCTTC	52
SKTI2.2 (reverse)	C terminal peptide	TCACACACTGCGAGAAAG	54

mide gel electrophoresis (PAGE) on gels treated with SDS, a mildly denaturing agent. Plant extracts from transgenic lines or from untransformed (control) plants were first incubated with trypsin for 15 min at 37°C to allow protease inhibition by recombinant SBTI. The samples were then fractionated into 10% (w/v) polyacrylamide gel slabs containing 0.1% (w/v) gelatin. The SDS-PAGE procedure is more fully described in Michaud et al. (1996). After electrophoretic migration at 4°C using the MiniProtean II electrophoretic unit (Bio-Rad, Richmond, CA, USA), the gels were transferred into a 2.5% aqueous solution of Triton X-100™ for 30 min at room temperature to allow proteinase (trypsin) renaturation. The gels were then placed in an activation or proteolysis buffer (50 mM tris, pH 8.0) at 37°C for 3 h and finally transferred into a protein staining solution [0.1% (w/v) Coomassie Brilliant Blue in 25% (v/v)/ 10% (v/v) acetic acid]. For storage, the gels were dried using Gel Drying Film (Promega, Madison, WI, USA). Trypsin activity was visualized as a clear (lysis) band against the blue gelatin background; trypsin inhibition was visualized as a weaker band compared with noninhibited controls, i.e., those with no plant extract and/or with extracts from nontransformed plants.

Results

Transformation

Calli were obtained in different parts of the explants, mainly at the cut edge of the petiole. Most positive calli were small, green, and round. After 2 mo in G24D medium, all calli proliferated. All established calli lines were confirmed GUS positive. Most developed calli were light green or yellowish, friable calli. Embryogenic orange calli were also obtained in Jonathan and Jewel. In regeneration media without auxins, a total of 50 transgenic lines have been obtained. Through somatic embryogenic regeneration of leaf segments, 22 transgenic lines of Maria Angola have been obtained.

GUS test of SKTI-4 transgenic lines

Good expression was observed in the 72 SKTI-4 lines of the 4 cultivars. Because GUS and SKTI-4 genes are driven by CaMV35S, it was possible to obtain positive reaction in different parts of the transgenic plants.

Kanamycin resistance evaluation of SKTI-4 transgenic lines

Eighteen lines of Jewel, PI-318846-3, Maria Angola, and Jonathan have been tested. All lines had the same performance in the medium with 100-mg/L kanamycin and without kanamycin, showing good calli formation. Untransformed leaf segments were necrotic in the presence of kanamycin in all cases. Figure 2 shows the response of Maria Angola's transgenic line and the negative control to the presence of kanamycin antibiotic in the medium.

The resistance to the antibiotic is due to the expression of the NPTII gene, which correlates with the expression of the inserted foreign gene. These lines are in propagation for bioassay (Table 2).

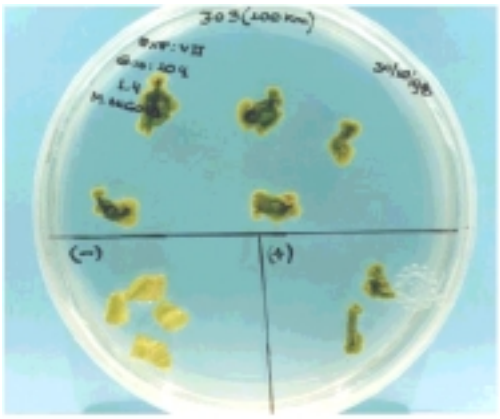

Figure 2. Kanamycin test. Calli formation on leaf segments of in vitro plant of transgenic line of cultivar Maria Angola in presence of high concentration of the antibiotic kanamycin (100 mg/L). Note dried segments and no calli formation of untransformed leaf segments of the same cultivar (negative control).

Table 2. List of transgenic clones with SKTI-4 gene ready for bioassay.

Name of transgenic lines ready for bioassay	
PI-318846-3 41 (1.1)	PI-318846-3 92 (1.3)
PI-318846-3 73 (1.1)	Jewel 6 (1.1)
PI-318846-3 89 (1.1)	Jonathan 3R (1.1)
PI-318846-3 89 (1.2)	Jonathan 21 (1.7)
PI-318846-3 89 (1.3)	M. Angola 63 (1.1)
PI-318846-3 89 (1.4)	M. Angola 104 (1.2)
PI-318846-3 89 (1.5)	M. Angola 104 (1.4)
PI-318846-3 89 (1.7)	M. Angola 104 (1.7)
PI-318846-3 92 (1.1)	M. Angola 106 (1.1)

Southern blot

To date eight transgenic lines of PI-318846-3 have been confirmed through Southern blot. Total DNA of transformed and untransformed control was digested with BamHI to release the internal fragment (653 bp) corresponding to the SKTI-4 cDNA. The SKTI-4 probe did not hybridize with DNA of the control plant (nontransgenic) but did with DNA of all PI-318846-3 transgenic lines tested, showing the insertion of the SKTI-4 gene in the transformants.

PCR analysis

The SKTI-4 gene was targeted with specific primers and amplified by PCR from DNA of in vitro transgenic plants. Twenty-four transgenic lines of Jewel, PI-318846-3, Maria Angola, and Jonathan have been confirmed for the insertion of SKTI-4 gene. The amplification band is shown for 16 samples (Figure 3).

Activity of recombinant SBTI

The inhibitory effect of recombinant SBTI on trypsin activity was analyzed in vitro with protein extracts from upper (youngest), middle, and bottom (oldest, nonsenescent) leaves of sweetpotato greenhouse plants (Figure 4). For each assay, the data were analyzed by comparing protease activities measured in the modified extracts with those measured in extracts from untransformed plants. That took into account the age-dependent activity of plant leaf endogenous protease and trypsin inhibitor activities (data not shown). Because the parental leaf material was identical for all clones analyzed, it could be assumed that the variations observed for a given stage of leaf development resulted

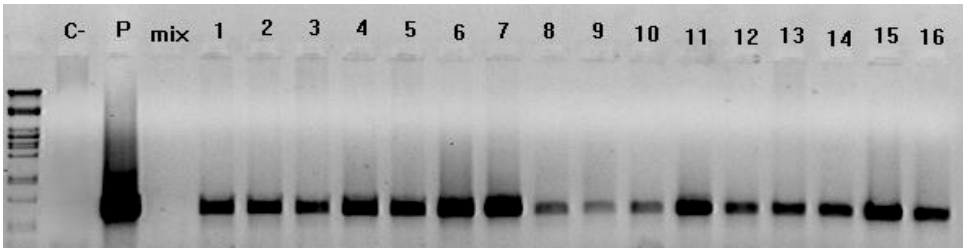

Figure 3. PCR amplification of 16 putative transgenic plants with SKTI-4 gene. The absence of the band in negative control (C-) and the band of the same weight (653 bp) in the positive control (P).

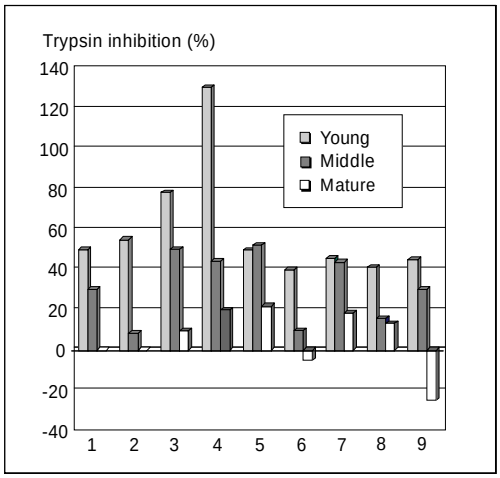

Figure 4. Differential expression of SBTI inhibitor in transgenic sweetpotato lines divided by young, middle, and mature leaves. Clones 1-8 are lines of transgenic PI-318846-3; Clone 9 (NT) is the nontransgenic control.

from various expression levels of the inserted DNA. As shown in Figure 4, a net increase in antitrypsin activity was noted for some clones regardless of leaf age (e.g., clones 3 and 4), when compared to the activity measured in the corresponding extracts obtained from nontransgenic clone 9. Collectively, these observations indicate the presence of *added* antitrypsin activity in the transgenic lines, due to the insertion and expression of the SBTI-encoding cDNA sequence into the sweetpotato genome.

Electrophoretic analysis

In parallel, mildly denaturing gelatin/SDS-PAGE allowed the occurrence of SBTI activity in extracts of transgenic plants expressing SKTI-4 to be seen. Because SBTI is a strong inhibitor of trypsin, the complex formed between the enzyme and the recombinant inhibitor may remain stable in the presence of SDS. That prevents restoration of trypsin activity during migration and allows the identification of transgenic lines expressing SBTI under an active form (Michaud et al., 1996). In the present case, noninhibited trypsin activities appeared as clear (lysis) bands against the blue gelatin background following electrophoresis.

Conversely, under the electrophoretic conditions used, the enzyme was *irreversibly* inactivated when mixed with an extract from a transgenic plant expressing active SBTI, resulting in no or only a weak lysis zone in the gel following gelatin/SDS-PAGE. Figure 5 shows the difference between the lysis bands obtained with extracts from the untransformed control and from two samples of transgenic lines with different levels of trypsin activity. Compared with the bands observed when trypsin was previously mixed with leaf extracts from the untransformed line, the bands observed with extracts from the transformed lines were weaker. The resulting intensities (and inhibitory levels) varied with the expression of the recombinant inhibitor.

Discussion

The present study was aimed at assessing the potential of an SBTI cDNA clone for the production of weevil-resistant sweetpotato plants. It is clear from both in vitro and electrophoretic assays that the transgenic lines possess increased antitrypsin activities compared with the parental control (nontransgenic) line. The most obvious increase in antitrypsin activity in the transgenic lines was noted for the upper

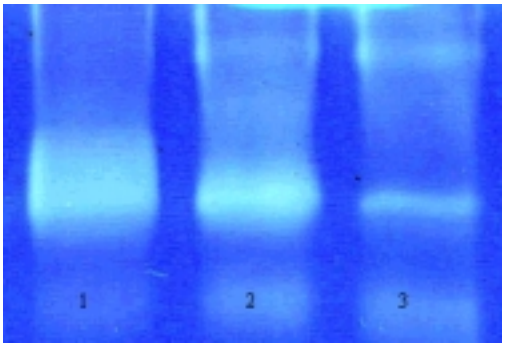

Figure 5. Electropherogram showing the gelatinolytic activity of trypsin preincubated with sweetpotato leaf extracts. Wells were loaded with 5 mg sweetpotato leaf proteins. Lane 1: untransformed control extract; lanes 2-3 PI-318836-3 transgenic lines extract.

(young) leaves (Figure 4, clone 4). Increased inhibitory activity was also noted for older leaves, suggesting the presence of recombinant SBTI in most of the leaves. Given the occurrence of SBTI-sensitive trypsin activity in sweetpotato weevils (Zhang et al., 1997), clones 3 and 4 (Figure 4) may represent interesting candidate lines for the development of sweetpotato weevil management strategies.

These two PI-318846-3 lines (89 1.3 and 89 1.4) are part of a group of transgenic lines expressing active SBTI that are in the multiplication process for bioassay. Continued efforts are needed to find the most appropriate combination of protease inhibitors, for enhancing the expression of plant defense proteins in sweetpotato, and for designing strategies to introduce protease inhibitor-expressing plants into integrated pest management systems.

Acknowledgements

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