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Regeneration of *Populus nigra* transgenic plants expressing a Kunitz proteinase inhibitor (*KTi*$_3$) gene

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Abstract

Transgenic poplar (*Populus nigra*, cv. Jean Pourtet) plants were recovered as a result of *Agrobacterium tumefaciens*-mediated transformation performed with EHA105 pBI-KUN strain. Plasmid pBI-KUN contains a 650 bp insert derived from the soybean (*Glycine max* L.) *KTi*$_3$, gene, coding for a Kunitz trypsin proteinase inhibitor. A total of 58 independent transgenic lines were obtained from 200 co-cultivated leaf explants. Southern blot hybridization analysis demonstrated the presence of *KTi*$_3$ gene in the poplar genome. Northern blot analysis of different kanamycin-resistant plantlets confirmed the accumulation of *KTi*$_3$ mRNA and revealed different levels of expression. The trypsin inhibitory activity was determined in poplar transgenic tissues by means of specific assay. Moreover, the trypsin-like digestive proteinases of the polyphagous moth *Lymantria dispar* (Lepidoptera, Lymantriidae) and *Clostera anastomosis* (Lepidoptera, Notodontidae) were detected and inhibited *in vitro* by Kunitz proteinase inhibitor from selected transgenic plants. Two insect bioassays were performed on *P. nigra* transgenic plant lines, using larvae of the above mentioned insects. In both cases larval mortality and growth as well as pupal weight were not significantly affected when the insects were fed on transgenic leaves and control leaves, respectively.

Abbreviations: BAP, 6-benzyl-aminopurine; IBA, indole-3-butyric acid; *KTi*, Kunitz trypsin inhibitor; LB, Luria Bertani; MS, Murashige & Skoog 1962; NAA, β-napthaleneacetic acid; SDS, sodium dodecyl sulfate; SSC, sodium chloride/sodium citrate; Tris, tris(hydroxymethyl)amino methane; WPM, woody plant medium.

Introduction

The genetic improvement of woody plants (forest, ornamental, plantation and fruit trees) has been limited by several factors, such as the size of the trees, the long life cycle and the lack of information concerning their basic genetics [28]. Genetic engineering provides the opportunity to transfer new specific traits of interest (for example, those for insect pest resistance) into valuable genotypes. Moreover, with the methods of genetic transfer, the period of time necessary to establish and analyse transgenic lines is generally shorter than the time required, by the tree, in order to reach sexual maturity and become suitable for classical breeding programmes [10]. Due to its small genome and ease of vegetative propagation, poplar (*Populus* species and hybrids) represents a useful model system for the investigation of the genetics and molecular biology of woody species [35]. The economic importance of poplar is now increasing, because of its fast growth and short rotation times; moreover, in the many countries of the temperate regions of the world, poplar trees represent the primary source of wood [15]. Poplars are seriously damaged by Lepidopteran pests

which cause heavy defoliations resulting in remarkably slower growth rates [23]. A severe damage to poplars is being caused by *Hyphantria cunea* Drury (Lepidoptera, Arctiidae), which was accidentally introduced into Europe by woody or other commercial material [1], due to the lack of natural enemies. Other recurrent poplar pests are represented by *Clostera anastomosis* L. (Lepidoptera, Notodontidae) and *Lymantria dispar* L. (Lepidoptera, Lymantriidae). In Italy, economic losses caused by defoliators are estimated to be 0.5% of the total woody production value [14]. Therefore, the protection of poplars against Lepidoptera is urgently needed.

Several chemically synthesized insecticides are currently used to control insect damage in agriculture but they are estimated to have an enormous cost worldwide and a strong environmental impact. The genetic engineering of insect tolerance into crop plants has become of significant interest to agricultural biotechnology. Ectopic expression of genes coding for proteinase inhibitors, which are part of the natural defence system developed by plants against insect attack, represent an effective approach. Successful protection against insect pests has been observed in different transgenic plants which contain genes coding for proteinase inhibitors [13, 16, 19, 25]. These proteins act by forming stoichiometric protein-protein complexes with various digestive enzymes, resulting in the competitive inhibition of their catalytic functions [24]. Four classes (cysteine, serine, metallo- and aspartyl-proteinase inhibitors) have been identified; serine proteinase inhibitors are active against trypsin and/or chymotrypsin-like enzymes [33]. Studies concerning the effects of proteinase inhibitors, localized in plant tissues or artificially introduced into diets, have shown that the native proteinase inhibitors can interfere with the growth and development of insects, by inhibiting their digestive enzymes [4, 8]. The soybean Kunitz factor is the most powerful trypsin inhibitor so far characterized in plants, and trypsin-like digestive enzymes have been identified in all Lepidoptera species examined [7, 20, 21, 22, 31, 38]. Recent progress in *Agrobacterium tumefaciens*-mediated gene transfer of black poplar [11, 12] makes it possible to produce new clones by introducing genes of agronomic interest. We used *A. tumefaciens*-mediated transformation of leaf explants to transform *P. nigra* L. with a Kunitz trypsin proteinase inhibitor gene.

Materials and methods

Plant materials and culture media

Aseptic shoot cultures of *Populus nigra* (cv. Jean Pourtet) were maintained *in vitro* on woody plant medium (WPM) [26] with or without IBA (0.5 mg/l), supplemented with 2% (w/v) sucrose (Merck), 0.3% (w/v) charcoal (Sigma) and 0.7% (w/v) agar-agar (Merck). Plantlets were propagated on the same basic medium, containing 0.5 mg/l IBA, through subculture of stem segments. Shoot regeneration was achieved by placing leaf explants on modified WPM (with MS microsalts), containing 0.4% (w/v) agar-agar and 0.2% (w/v) gelrite (Schweizerhall), supplemented with 0.5 mg/l BAP and 0.05 mg/l NAA. All cultures were maintained in a growth chamber at 22–25 °C, with a 16:8 h light/dark cycle.

Bacterial strains and plasmids

The *Agrobacterium tumefaciens* strain EHA105 [17], a non-oncogenic derivative of strain A281, which harbours the hypervirulent helper Ti plasmid pTiBo542 was used for transformation. The pBI-KUN vector plasmid (11 kb, Figure 1A) contains a 650 bp fragment that was obtained by PCR amplification of the soybean KTi_3 gene [18] using two specific primers carrying a *Bam*HI site (K_1: GGATCCATGAAGAGCACCATCTTCTTTCTCTTTC) and a *Pvu*II site (K_2: GTACCGGAAAGAGCGTCACTCACTGTCGAC). The amplified fragment was cloned into pGEM-T (Promega), sequenced and then introduced into the binary vector pBI121 (Clontech), routinely used for plant transformation, under the control of the cauliflower mosaic virus (CaMV) 35S promoter and the polyadenylation region from the nopaline synthase gene (*nos-ter*) [2]. The *Pvu*II site was lost during the cloning procedure. The resulting binary plasmid, pBI-KUN, was subsequently transferred to *A. tumefaciens* by electroporating at 2500 V an EHA105 suspension culture grown overnight and washed with 10% glycerol. Bacterial cultures were grown overnight in LB [34] containing 150 mg/l kanamycin (Sigma) and 150 mg/l rifampicin (Lepetit) and resuspended in liquid WPM to a final density of 0.35–0.6 (A_{550}).

Transformation and plant regeneration

Transformation and regeneration conditions were those described by Confalonieri *et al.* [12]. Leaf explants

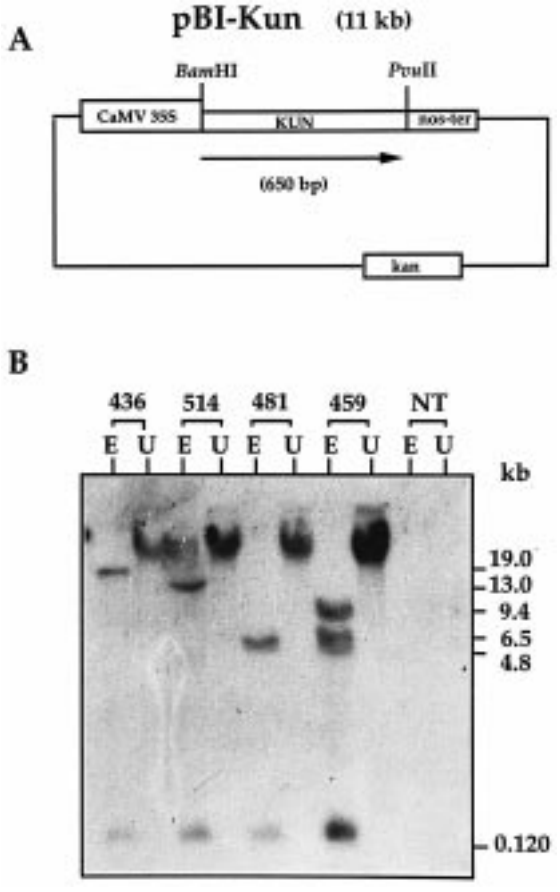

Figure 1. A. Schematic representation of the binary plasmid pBI-KUN. CaMV 35S, cauliflower mosaic virus 35S promoter; KUN, *KTi*$_3$ gene sequence; nos-ter, nopaline synthase gene terminator; kan, neomycin phosphotransferase-coding sequence. B. Southern blot analysis of genomic DNAs extracted from four different transgenic poplar lines (KUN436, KUN514, KUN481, KUN459) following transformation with EHA105 pBI-KUN *A. tumefaciens* strain. *KTi*$_3$ was used as a probe for Southern blot hybridization. U, high-molecular-weight undigested DNA. E, *Eco*RI-digested DNA; NT, untransformed control.

were pre-cultured for 24 h on semi-solid regeneration medium, then immersed for 20 min in 25 ml of a liquid culture (containing 200 μM acetosyringone, Aldrich) of EHA105 pBI-KUN strain. The bacterial suspension was omitted in control plates. After blotting with sterile filter paper, the explants were co-cultivated for 48 h on the same medium. Following co-cultivation, the inoculated explants were rinsed with sterile distilled water containing 1 g/l cefotaxime (Hoechst) and 1.5 g/l carbenicillin (Pfizer), placed on selective regeneration medium (100 mg/l kanamycin, 300 mg/l carbenicillin and cefotaxime), and incubated in the dark for 7 days. Leaf explants were subcultured every 10–15 days on selective regeneration medium for the first 40–45 days. Control explants were cultured using the same conditions described for the co-cultivated leaf explants. The regenerating calli were excised from the primary explants, cultured on the same medium for 30 days, and then transferred to selective regeneration medium containing a reduced amount of BAP (0.05 mg/l). Shoots (sized 1–2 cm) were elongated on WPM in the absence of growth regulators and kanamycin. Subsequently, they were subcultured twice on WPM containing 100 mg/l kanamycin to test rooting. Only those plantlets which rooted under these conditions were considered kanamycin-resistant and transferred to the greenhouse. Cultures were kept at 22–25 °C, with a 16:8 h light/dark cycle. Transformation frequency was defined as percentage of inoculated leaf explants that produced kanamycin-resistant plantlets.

Southern blot hybridization analysis of plant genomic DNA

Total DNA was extracted from leaves of untransformed and putatively transgenic plants regenerated from independent transformation events, according to Rogers and Bendich [32], by freezing and grinding the tissues in liquid N_2. For each sample, 10 μg of genomic DNA were digested with *Eco*RI (Promega), according to the manufacturer's instructions. DNA fragments were separated on 0.8% (w/v) agarose gels (Sigma) and subsequently transferred onto nylon membranes (Boehringer Mannheim), following the supplier's instructions. Membranes were prehybridized for 1–2 h at 42 °C in 50% (v/v) formamide, 5× SSC, 0.5% SDS, 5× Denhardt's solution, 100 mg/ml sonicated salmon sperm DNA. The purified *Bam*HI/*Pvu*II fragment (650 bp), containing the complete coding sequence of the *KTi*$_3$ gene, was labelled with RTS RadPrime DNA Labeling System (Gibco-BRL) and used as probe. Hybridization was carried out for 16 h, under the same conditions described for prehybridization. Membranes were washed twice with 2× SSC, 0.1% SDS (10 min) at room temperature and once with 1× SSC, 0.1% SDS at 65 °C (15 min) and subsequently autoradiographed with Hyperfilm-MP (Amersham) at −70 °C for 4–6 days.

Total RNA extraction and northern blot hybridization analysis

Total RNA was isolated from leaves of untransformed and putative transgenic poplar plantlets using 'Plant

RNA-A Extract' kit (Clontech) to determine the relative amounts of *KTi*$_3$ related RNA. Five μg of total RNA were separated on 1.5% (w/v) agarose denaturing formaldehyde gels (SeaKem GTG agarose, FMC BioProducts), and the RNA was visualized by staining the gels with ethidium bromide (1 mg/ml). The separated RNAs were subsequently blotted to nylon membranes (Hybond-N, Amersham), according to manufacturer's instructions, and cross-linked by UV irradiation. Prehybridization and hybridization were performed as for the Southern blot analysis.

Assays of proteinase inhibition

Leaves from untransformed and selected transgenic plantlets were homogenized in 10 mM Tris (pH 8.0) and centrifuged at 4 °C for 20 min at 13 000 $\times$ g. Samples were then diluted to a standard protein concentration (800 μg/ml), according to Bradford [5]. The trypsin inhibitory activity was assayed as follows. The plant extract solution (100 μl) was mixed with 80 μl of trypsin solution (100 μg/ml, Sigma), and the final volume was brought to 300 μl with 0.05 mM Tris-HCl buffer (pH 7.5) containing 0.02 mM $CaCl_2$ and preincubated at 30 °C for 10 min. One milliliter of 1 mM benzoyl-L-arginine *p*-nitroanilide (L-BApNA, Sigma) solution in the same buffer was added to the mixture and the sample was incubated at 30 °C for 10 min. Then 200 μl of a 30% acetic acid solution was added to stop the reaction. The inhibitory activity was calculated by comparing the variation in absorbance at 405 nm before ($T = 0$) and after ($T = 10$) the incubation step, with a scale ($R^2 = 0.991$) obtained replacing the plant protein extract with different amounts (0–10 μg) of purified trypsin inhibitor (Sigma), and referred to μg of inhibitor per gram leaf fresh weight. The average absorbance of control samples was used as instrumental base line. Two different experiments were performed and, for each of them, three replications were used. Data from both experiments were subjected to a combined analysis of variance using STAT-ITCF software (ITCF, France). LSD value was calculated and significant differences between means were determined using the Student-Newman-Keuls Multiple Range Test at the $\alpha = 0.01$ level.

In vitro inhibition studies were conducted using the protein extracts obtained from *L. dispar* and *C. anastomosis* midguts and the *KTi*$_3$ inhibitor solutions from selected transgenic plants (lines KUN436 and KUN459, respectively). Untransformed plants were used as a control. For each poplar line, three independent protein extracts (100 μl, 800 μg/ml) were analyzed. Assays of proteinase inhibition were carried out as described above, replacing the trypsin solution with the midgut protein solution (100 μg/ml trypsin-like proteinases). Residual activities were calculated by spectrophotometric assays and expressed as percentages of the mean activity of the control samples.

Proteinase activity in L. dispar *and* C. anastomosis *guts*

Forty guts were surgically removed from third-instar larvae, transferred to centrifuge tubes containing 0.15 M NaCl, homogenized and then centrifuged at 4 °C for 15 min at 13 000 $\times$ g [25]. The clear part of the supernatant, representing the midgut protein solution, was diluted to a standard protein concentration (600 μg/ml), according to Bradford [5]. Two independent samples were analyzed for each insect $\times$ proteinase substrate combination. The trypsin activity was assayed by adding 80 μl of midgut protein solution to 640 μl (43 μg/ml) of the chromogenic substrate *N*-benzoyl-L-arginine *p*-nitroanilide (L-BApNA) diluted in 50 mM Tris-HCl buffer (pH 8.0). Reaction tubes were incubated at 30 °C for 20 min before spectrophotometric assay. The total proteinase activity was assayed by adding 50 μl of midgut protein solution to 100 μl of a 2% (w/v) azocasein diluted in 50 mM potassium phosphate buffer (pH 6.8). The test was performed at 37 °C for 30 min. After this period, the reaction was stopped using 480 μl of 10% (v/v) trichloracetic acid solution. Samples were kept on ice for 15 min and then centrifuged for 3 min at 8000 $\times$ g. The supernatant was collected and 500 μl of supernatant was mixed with 580 μl of 1 M NaOH before spectrophotometric assay. The trypsin and total proteinase activities were measured at 450 nm and compared to standard curves ($R^2 > 0.98$) obtained using different amounts of pure trypsin (0–10 μg) and papain (0–20 μg, Sigma). Results were expressed as a percentage of trypsin or total proteinases relative to the total protein content of the midgut.

Bioassays of transgenic poplar plants for insect resistance

An insect bioassay was performed on *P. nigra* transgenic plant lines using larvae of the polyphagous moth *L. dispar*. A stabilized insect strain was used, reared under laboratory conditions since 1990 at the Poplar Research Institute-Casale M.to (Italy) on ICN Gypsy

Moth Diet (ICN Biochemicals). Transgenic and untrasformed control plantlets were transferred to pots and grown in a greenhouse. Four fully developed leaves on plants of five different transgenic lines (KUN436, KUN455, KUN459, KUN481 and KUN514, respectively) expressing the Kunitz proteinase inhibitor, and two untransformed control lines, were confined in single Petri dishes with net cover. Ten newly moulted second-instar larvae were collected by a soft, thin brush and introduced into each Petri dish. A total of 40 larvae, divided into 4 replications, were used for each tested line. Because each replication contained larvae coming from single egg batch, they have been considered as blocks. After one week, the larvae were transferred to new Petri dishes, kept in a growth chamber (25 °C, 16:8 h light/dark cycle) and fed with detached leaves coming from the same selected plants. Leaf petioles were inserted through a parafilm cap into small vials containing distilled water. Leaves were replaced every 2 days. The feeding, development and mortality rates of larvae before pupation, and pupal weights, were recorded. The results were subjected to analysis of variance (randomized complete block design) for statistical evaluation of differences among treatments. Data concerning larval mortality were $\arcsin\sqrt{x}$ transformed before statistical analysis. In case of significant treatment effect, a Student-Newman-Keuls Multiple Range Test was carried out in order to identify pairwise significant differences among treatments.

A second insect bioassay was carried out on three transgenic lines (KUN436, KUN455, KUN514) and two untransformed control lines using larvae of *C. anastomosis* reared in an outdoor entomological cage since 1995. Egg batches were collected and transferred to the laboratory. After hatching, the young larvae were fed with poplar leaves until moulting. For each line, 40 second-instar larvae, divided into 4 replications of ten each, were introduced to Petri dishes and fed with leaves detached from the selected plants. All the other conditions, as well as the variables measured, did not differ from the previous test.

Results

Transformation and plant regeneration

Fifty-eight putative transgenic plant lines were obtained from 200 co-cultivated leaf explants with a transformation frequency of 29%. No callus formation or shoot organogenesis was observed in control leaf explants cultured on selective regeneration medium. Putative transgenic plantlets were *in vitro* propagated and then placed in a greenhouse to collect the amounts of tissues required to perform molecular and biochemical analyses and insect bioassays.

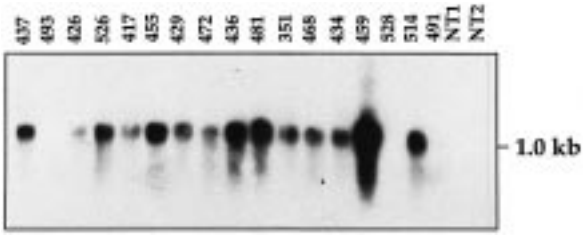

Figure 2. Northern blot analysis of equally loaded total RNAs extracted from different transgenic poplar lines regenerated following transformation with *A. tumefaciens* EHA105 pBI-KUN strain, using the 650 bp *KTi*$_3$ gene sequence as a probe. Lanes NT1 and NT2: untransformed controls.

Molecular analyses of transgenic plants

The presence of the *KTi*$_3$ proteinase inhibitor gene in different putative transformed plant lines was tested by Southern blot hybridization analysis. For each of the tested lines, undigested and *Eco*RI-digested DNAs were analyzed using the *KTi*$_3$ fragment as molecular probe. Results of this experiment are reported in Figure 1B. The presence of two *Eco*RI sites in the *KTi*$_3$ gene sequence resulted in the production of a small (120 bp) hybridizing *Eco*RI fragment. Different hybridization bands appeared in lanes containing *Eco*RI-digested DNA, indicating the occurrence of distinct integration events. Moreover, hybridization signals were located in the high molecular weight fraction of undigested DNAs, thus confirming the stable integration of the *KTi*$_3$ gene sequence in the tested lines. No signals were detected using DNA from the untransformed control plant.

Northern blot hybridization analysis using the *KTi*$_3$ probe detected a single signal of ca. 1 kb in most of the putative transgenic plantlets (Figure 2). The transcript showed the expected size, with a large variation in the steady-state level in different plant lines. No signal was detected in control plants. Northern blot analysis showed that transformants KUN459, KUN436, KUN481, KUN459 and KUN514 produced the strongest signals, and therefore, these lines were tested for their trypsin inhibitory activity.

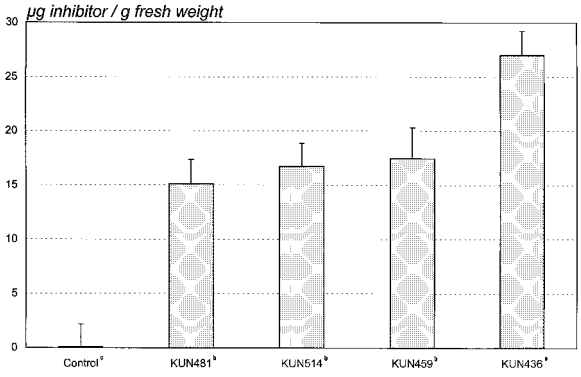

Figure 3. Amount of Kunitz proteinase inhibitor in different control and transgenic poplar lines transformed with EHA105 pBI-KUN *A. tumefaciens* strain. The above data are the mean values obtained from two experiments, each with three replications. Means with the same letter are not significantly different at 1% by the Student-Newman-Keuls Multiple Range Test. The vertical bars indicate the standard error.

Proteinase inhibitor expression

The leaf extract from all selected transgenic lines revealed a trypsin inhibitory activity higher than the control (Figure 3). The combined analysis of variance showed a significant difference ($p < 0.0001$) between the treatments. An LSD value was determined and corresponded to 6.58 μg of proteinase inhibitor per gram of fresh tissue. Student-Newman-Keuls Multiple Range Test revealed significant differences ($p < 0.001$) among KUN436[a] and KUN459[b], KUN514[b], KUN481[b] and the control[c]. A 27 μg portion of proteinase inhibitor per gram of fresh tissue was detected in the best transgenic poplar line. At the highest levels of gene expression, Kunitz represented more than 1% of the total soluble proteins.

Protein extracts from selected transgenic poplar lines were analysed to measure the ability of Kunitz proteinase inhibitor to inhibit trypsin activity from the midgut of *L. dispar* and *C. anastomosis*. Plant extracts from KUN436 and KUN459 transgenic lines caused 66% and 43% average reduction in the gypsy moth trypsin activity, respectively. Using protein extracts from the same transgenic poplar lines, the average decrease in *C. anastomosis* trypsin activity was 73% and 49%, respectively. In contrast, no inhibitory effects on *L. dispar* and *C. anastomosis* intestinal trypsin-like enzymes were recorded using untransformed poplar plants.

Proteinase activity in L. dispar *and* C. anastomosis *guts*

Trypsin and total proteinase activities were determined in the *L. dispar* and *C. anastomosis* larval midguts. Trypsin activity was observed in the gypsy moth midguts using L-BApNA as substrate. The percentage of trypsin on the midgut total proteins was 13% in both samples. Using azocasein as a substrate, the percentage of total proteinases relative to midgut total proteins was only 6% higher (19% in both samples). In *C. anastomosis*, total proteinases represented about 9% of total midgut proteins, and the percentage of trypsin-like enzymes corresponded to 5%. Therefore, trypsin-like proteinases represent the most important class of digestive enzymes in these insects.

Insect bioassays

The results of the bioassay carried out on *L. dispar* larvae are reported in Table 1. Larval mortality was mainly observed in the first week of treatment and after pupation. Some uncontrolled mortality was found after manipulation of the larvae during the test as well as during our previous experiences (unpublished data), causing high variability among replications, aside from block effects, which prevented any statistical evidence for differences among the treatments ($p = 0.6209$). Among the variables, only the male pupal weight showed a significant probability level ($p = 0.0095$), KUN514 being significantly higher in comparison to the other lines according to Student-Newman-Keuls Multiple Range Test ($p = 0.05$). No significant correlation was found between larval mortality and either male pupal weight ($r = 0.178$) or female pupal weight ($r = 0.138$). At visual inspection, the consumed leaf area appeared rather homogeneous among the transgenic and the control lines. The test performed on *C. anastomosis* gave almost the same results as the previous one (Table 2). Manipulation did not disturb larval performances and the error variance was much lower. No statistical evidence supported a hypothesis of inhibitory activity of the KTi_3 transgenic lines. Also in this case, only for male pupal weight was the treatment's F significant ($p = 0.0182$), KUN436 resulting significantly higher than the other lines according to Student-Newman-Keuls Multiple Range Test ($p = 0.05$). In no ANOVA were the within-treatment variances significantly heterogeneous.

Table 1. Results of a bioassay carried out on *Lymantria dispar* larvae fed with leaves of five different *KTi*$_3$ transgenic poplar lines.

Poplar line	Larval weight (mg)	Larval mortality (%)	Male pupal weight (mg)	Female pupal weight (mg)
KUN436	239.6	21.1	254.7^{b*}	512.7
KUN455	329.6	35.9	277.7^{b}	666.6
KUN459	261.7	20.0	270.1^{b}	522.3
KUN481	313.6	16.7	265.1^{b}	526.2
KUN514	348.2	32.5	359.7^{a}	662.0
Control 1	348.0	2.6	294.3^{b}	584.6
Control 2	356.0	8.3	306.2^{b}	721.2
F	0.43	0.75**	4.16	2.22
(df)	(6)	(6)	(6)	(6)
Probability	0.8532	0.6209	0.0095	0.0942
Pooled SE	140.5	22.48	34.8	112.62

* means with the same letter are not significantly different at 5% by the Student-Newman-Keuls Multiple Range Test.
** data were arcsin$\sqrt{x}$ transformed before analysis of variance.

Table 2. Results of a bioassay carried out on *Clostera anastomosis* larvae fed with leaves of three different *KTi*$_3$ transgenic poplar lines.

Poplar line	Larval weight (mg)	Larval mortality (%)	Male pupal weight (mg)	Female pupal weight (mg)
KUN436	130.7	2.5	205.6^{a*}	338.0
KUN455	124.2	0.0	176.3^{b}	323.7
KUN514	143.8	5.0	181.1^{b}	280.1
Control 1	115.9	0.0	180.3^{b}	308.2
Control 2	127.8	2.5	178.8^{b}	300.4
F	2.73	0.84**	4.56	2.84
(df)	(4)	(4)	(4)	(4)
Probability	0.0790	0.5273	0.0182	0.0717
Pooled SE	12.35	8.41	11.22	26.28

* means with the same letter are not significantly different at 5% by the Student-Newman-Keuls Multiple Range Test.
** data were arcsin $\sqrt{x}$ transformed before analysis of variance.

Discussion

Transgenic poplar plants (*P. nigra* cv. 'Jean Pourtet'), containing a Kunitz proteinase inhibitor gene from a dicot plant, soybean, were regenerated after co-cultivation with *A. tumefaciens* EHA105 pBI-KUN strain. A total of 58 independent transgenic lines were produced with a transformation efficiency of 29%. Southern blot hybridization analysis was performed on DNA extracted from leaves of regenerated plantlets, using the *KTi*$_3$ gene coding sequence as probe. The analysis produced positive results for each poplar line tested, thus indicating the presence of the foreign *KTi*$_3$ sequence in the plant genome. The northern analysis clearly showed that the target gene was expressed at high levels. The trypsin inhibitor activity of the Kunitz proteinase inhibitor was more than 1% of the total soluble proteins. This value is close to that found in seeds of different leguminous plants [30], and is similar to the one recorded in tobacco plants transformed with

the same pBI-KUN construct [27]. A 27 μg portion of proteinase inhibitor per gram of fresh tissue was detected in the best transgenic poplar line. The trypsin-like and total digestive proteinase activities of *L. dispar* and *C. anastomosis* larvae were determined. Trypsin-like proteinases were detected in the midguts of both insects and represented the most abundant class of their digestive enzymes. Similar observations have been reported for *L. dispar* by Valaitis [38]. We have shown that the Kunitz proteinase inhibitor (KTi_3) in transgenic poplar plants was a good inhibitor of trypsin digestive proteinases of *L. dispar* and *C. anastomosis* larvae. However, insect bioassays performed on *L. dispar* and *C. anastomosis* larvae did not provide any evidence of increased insect pest resistance in transgenic poplar plants. Leaves from different transgenic lines expressing the trypsin inhibitor gene did not affect feeding and larval growth, when compared to leaves from untransformed poplar plantlets. A different performance was shown by tobacco transgenic plants transformed with the same pBI-KUN construct, which proved to be resistant to the cotton leafworm (*Spodoptera littoralis*), causing high larval mortality [27]. These results seem to contradict the data from our experiments in which the trypsin-like enzymes identified in the midgut of *L. dispar* and *C. anastomosis* larvae were inhibited, *in vitro*, by purified trypsin proteinase inhibitors, and by protein extracts from transgenic poplars. The causes of the failure of Kunitz proteinase inhibitor to affect larval growth and mortality in poplar could be found in several factors. The very humid microclimate of Petri dishes, where larvae were fed, could have somewhat prevented larval dehydration, which is a co-causal factor of mortality after consumption of proteinase inhibitors (S. Marchetti, personal communication). The residual proteinase activity in *L. dispar* and *C. anastomosis* could be sufficient to support an almost normal growth. However, an intriguing explanation might be represented by the loss in ability of Kunitz proteinase inhibitor to block trypsin activity of larvae fed with transgenic leaves. Recently Broadway [6] and Bolter and Jongsma [3] evaluated the susceptibility of serine and cysteine proteinase extracted from Lepidoptera and Coleoptera to serine and cysteine proteinase inhibitors. The predominant digestive enzymes in the midgut of larvae reared on artificial diet supplemented with proteinase inhibitors were resistant to inhibition by the same inhibitors, thus contradicting the marked inhibition of proteinase activity detected in larvae fed on artificial substrate in the absence of proteinase inhibitor. This finding suggests that insects could dynamically modify the spectra of their digestive enzymes. Although *in vitro* analyses allowed the identification of specific proteinase inhibitors than can successfully be employed for the control of specific insects [9], our study indicated that Kunitz soybean inhibitor is an ineffective feeding deterrent against *L. dispar* and *C. anastomosis* insects. To date, this is the first demonstration of proteinase inhibitor gene expression in transgenic *Populus nigra* plants at the molecular and biochemical levels. However, although no insect pest resistance was obtained, the successful transformation and expression of different proteinase inhibitors into other poplar species [25] and crops such as tobacco [27], cotton [37], alfalfa [36] and rice [13] shows the interesting perspectives by this powerful strategy offered in breeding programmes.

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References

1. Allegro G: Un nemico nuovo per la pioppicoltura italiana: l'ifantria americana (*Hyphantria cunea* Drury). Econ Mont 19: 50–53 (1987).
2. Barton KA, Whiteley HR, Yang NS: *Bacillus thuringiensis* δ-endotoxin expressed in transgenic *Nicotiana tabacum* provides resistance to lepidopteran insects. Plant Physiol 85: 1103–1109 (1987).
3. Bolter CJ, Jongsma MA: Colorado potato beatles (*Leptinotarsa decemlineata*) adapt to proteinase inhibitors induced in potato leaves by methyl jasmonate. J Insect Physiol 41: 1071–1078 (1995).
4. Boulter D: Insect control by copying nature using genetically engineered crops. Phytochemistry 34: 1453–1466 (1993).
5. Bradford MM: A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248–254 (1976).
6. Broadway RM: Are insect resistant to plant proteinase inhibitors? J Insect Physiol 41: 107–116 (1995).
7. Broadway RM, Duffey SS: The effect of dietary protein on the growth and digesty physiology of larval *Heliothis zea* and *Spodoptera exigua*. J Insect Physiol 32: 673–680 (1986).
8. Christeller JT, Laing WA, Markwick NO, Burgess EPJ: Midgut protease activities in 12 phytophagous lepidopteran larvae:

dietary and protease inhibitor interactions. Insect Biochem Mol Biol 22 (7): 735–746 (1992).
9. Christeller JT, Shaw BD: The interaction of a range of serine proteinase inhibitors with bovine trypsin and *Costelytra zealandica* trypsin. Insect Biochem 19: 233–241 (1989).
10. Chupeau MC, Lemoine M, Chupeau Y: Requirement of thidiazuron for healthy protoplast. Development to efficient tree regeneration of a hybrid poplar (*Populus tremula* × *P. alba*). Plant Physiol 141: 601–609 (1993).
11. Confalonieri M, Balestrazzi A, Bisoffi S: Genetic transformation of *Populus nigra* by *Agrobacterium tumefaciens*. Plant Cell Rep 13: 256–261 (1994).
12. Confalonieri M, Balestrazzi A, Bisoffi S, Cella R: Factors affecting *Agrobacterium tumefaciens*-mediated transformation in several black poplar clones. Plant Cell Tissue Organ Cult 3: 215–222 (1995).
13. Duan X, Li X, Xue Q, 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).
14. ENCC: Insetti parassiti del pioppo. ENCC, Rome (1992).
15. FAO: Poplars and willows in wood production and land use. FAO Forestry Series No. 10. FAO, Rome (1980).
16. Hilder VA, Gatehouse AMR, Sherman SE, Barker RF, Boulter D: A novel mechanism of insect resistance engineered in tobacco. Nature 330: 160–163 (1987).
17. Hood EE, Gelvin SB, Melchers LS, Hoekema A: New Agrobacteria helper plasmids for gene transfer to plants. Transgen Res 2: 208–218 (1993).
18. Jofuku KD, Goldberg RB: Kunitz trypsin inhibitor genes are differentially expressed during the soybean life cycle and in transformed tobacco plants. Plant Cell 1 (11): 1079–1093 (1989).
19. Johnson R, Narvaez J, An G, Ryan C: Expression of proteinase inhibitors I and II in transgenic tobacco plants: effects on natural defense against *Manduca sexta* larvae. Proc Natl Acad Sci USA 86: 9871–9875 (1990).
20. Johnston KA, Gatehouse JA, Anstee JH: Effects of soybean protease inhibitors on the growth and development of larval *Helicoverpa armigera*. J Insect Physiol 39: 657–664 (1993).
21. Johnston KA, Lee MJ, Brough C, Hilder VA, Gatehouse AMR, Gatehouse JA: Protease activities in the larval midgut of *Heliothis virecens*: evidence for trypsin and chymotrypsin-like enzymes. Insect Biochem Mol Biol 25: 375–383 (1995).
22. Jongsma MA, Peters J, Stiekema WJ, Bosch D: Characterization and partial purification of gut proteinases of *Spodoptera exigua* Hubner (Lepidoptera: Noctuidae). Insect Biochem Mol Biol 26: 185–193 (1996).
23. Kulman HL: Effects of insect defoliation on growth and mortality of trees. Annu Rev Entomol 16: 289–316 (1971).
24. Laskowski M, Kato I: Protein inhibitors of proteinases. Annu Rev Biochem 49: 593–626 (1988).
25. Leplé JC, Bonadé Bottino M, Augustin S, Pilate G, Dumanois Le Tan V, Delplanque A, Cornu D, Jouanin L: Toxicity to *Chrysomela tremulae* (Coleoptera: Chrysomelidae) of transgenic poplars expressing a cysteine proteinase inhibitor. Mol Breed 1: 319–328 (1995).
26. Lloyd G, McCown BH: Commercially feasible micropropagation of mountain laurel, *Kalmia latifolia*, by use of shoot-tip culture. Proc Int Plant Prot 30: 421–427 (1991).
27. Marchetti S, Giordano A, Chiaba C, Pfeiffer A, Delledonne M, Fogher C, Savazzini F, Cervini P, Bassi C, Chiesa F, Reguzzi MC, Cravedi P: Genetic engineering of plants for insect resistance using proteinase-inhibitor genes. Proc IPBA 113–119 (1994).
28. McCown BH, McCabe DE, Russel DR, Robison DJ, Barton KA, Raffa KF: Stable transformation of *Populus* and incorporation of pest resistance by electric discharge particle acceleration. Plant Cell Rep 9: 590–594 (1991).
29. Murashige T, Skoog F: A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15: 73–97 (1962).
30. Norioka N, Hara S, Ikenaka T, Abe J: Distribution of the Kunitz and the Bowman-Birk family proteinase inhibitors in leguminous seeds. Agric Biol Chem 52: 1245–1252 (1988).
31. Purcell JP, Greenplate JT, Sammons RD: Examination of midgut luminal proteinase activity in six economically important insects. Insect Biochem Mol Biol 22: 41–47 (1992).
32. Rogers SO, Bendich AJ: Extraction of DNA from plant tissues. In: Gelvin SB, Schilperoort RA (eds) Plant Molecular Biology Manual A6: 1–10. Kluwer Academic Publishers, Dordrecht, Netherlands (1988).
33. Ryan CA: Proteinase inhibitor gene families: strategies for transformation to improve plant defense against herbivores. Bioessays 10: 20–24 (1989).
34. Sambrook J, Fritsch EF, Maniatis T: Molecular Cloning: A Laboratory Manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY (1989).
35. Subramaniam R, Reinold S, Molitor EK, Douglas CJ: Structure, inheritance and expression of hybrid poplar (*Populus trichocarpa* × *Populus deltoides*) phenylalanine ammonia-lyase genes. Plant Physiol 102: 71–83 (1993).
36. Thomas JC, Wasmann C, Echt C, Dunn RL, Bohnert HJ, McCoy TJ: Introduction and expression of an insect proteinase inhibitor in alfalfa (*Medicago sativa* L.). Plant Cell Rep 14: 31–36 (1994).
37. Thomas JC, Adams DG, Keppenne VD, Wasmann C, Brown JK, Kanost MR, Bohnert HJ: Protease inhibitors of *Manduca sexta* expressed in transgenic cotton. Plant Cell Rep 14: 758–762 (1995).
38. Valaitis AP: Gypsy moth midgut proteinases: purification and characterization of luminal trypsin, elastase and the brush border membrane leucine aminopeptidase. Insect Biochem Molec Biol 25 (1): 139–149 (1995).