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Transformation of white poplar (*Populus alba* L.) with a novel *Arabidopsis thaliana* cysteine proteinase inhibitor and analysis of insect pest resistance

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Abstract

Transgenic white poplar *(Populus alba* L.) plants expressing a novel *Arabidopsis thaliana* cysteine proteinase inhibitor (*Atcys*) gene have been produced using *Agrobacterium tumefaciens*-mediated gene transfer. Internodal stem segments of cv. Villafranca were co-cultivated with the EHA105 pBI-Atcys *A. tumefaciens* strain. Sixteen putative transgenic plant lines were regenerated from different calli with a transformation efficiency of 11%. The integration and expression of the cysteine proteinase inhibitor *(Atcys)* gene into the plant genome was confirmed by Southern and northern blot analyses. Papain inhibitory activity was detected in poplar transgenic tissues by means of a specific *in vitro* assay. Such activity was sufficient to inhibit most of the digestive proteinase activity of chrysomelid beetle *(Chrysomela populi* L.) and confer resistance to *C. populi* larvae on selected transgenic plants. A close correspondence between the inhibition of papain and resistance to poplar leaf beetle was observed in all tested transgenic lines. Our results indicate that *Atcys* could be succesfully employed in breeding programmes aimed at the selection of new poplar genotypes resistant to major insect pests.

Abbreviations: BAP – 6-benzyl-aminopurine, CIM – callus inducing medium, IBA – indole-3-butyric acid, LB – Luria-Bertani, MS – Murashige & Skoog 1962, TDZ – Thidiazuron, WPM – woody plant medium

Introduction

White poplar *(Populus alba* L.) occupies a great geographic range which includes the Centre and South of Europe, North Africa, Western and Central Asia (FAO 1980). This species is mainly used for reforestation in floodplains along river courses and in the specialized stands for the production of sawlogs. Lepidopteran and coleopteran pests are a constant threat to poplars. Lepidoptera can cause heavy defoliations, leading to reduced wood production (Kulman 1971). Coleoptera are definitely the most important pests in Italy, causing a decrease in wood quality or defoliations which result in slower growth rates (ENCC 1992).

Chrysomelid beetles are very injurious, mainly in nurseries, because they not only reduce the size and value of commercial stock, but also prevent proper lignification of the apical shoot of plantlets, that are subject to necrosis at transplanting (Allegro and Giorcelli 1997). Widespread attacks of *Chrysomela populi* L. on poplar nurseries are almost yearly recurrent in Italy, and many plantations are sprayed with synthetic insecticides having a deleterious environmental impact. Introduction of genes conferring pest resistance

from wild species into commercial genotypes by classical breeding methods require a prolonged period of time due to the long life cycle of these species. Recently, transfer of foreign genes into poplars by genetic engineering techniques has become routine in several laboratories (Jouanin et al. 1993). This technology provides an opportunity to modify and improve selected genotypes for important agronomic traits, such as resistance to insect pests, within a short period of time. A novel approach to control insect predation involves the use of proteinase inhibitor-coding sequences and their introduction and expression into the poplar genome. The physiological role of proteinase inhibitors in plants has been the subject of extensive research over several years, since they are part of the natural plant defence system against a wide spectrum of insect pests (Ryan 1990). Four classes of proteinase inhibitors (cysteine, serine, metallo- and aspartyl proteinase inhibitors) have been identified. Several families of Coleoptera and Hemiptera possess cysteine proteinases as part of their digestive systems, which appear to play important roles in the digestion process of food proteins (Ryan 1990). Previous studies on the effects of both natural and synthetic cysteine proteinase inhibitors have shown that they may inhibit digestive cysteine proteinases, thus limiting the availability of amino-acids for insect growth and development (Ryan 1990). Genes encoding a number of proteinase inhibitors have already been isolated and characterized (Abe et al. 1987, 1992; Gruden et al. 1997). Significant levels of resistance to the poplar leaf beetle (*Chrysomela tremulae*) have been achieved in transgenic *Populus tremula* × *Populus tremuloides* using a cysteine proteinase inhibitor from rice (Leplé et al. 1995). Transgenic poplar lines have been described expressing genes coding for different serine proteinase inhibitors (Heuchelin et al. 1997; Confalonieri et al. 1998) or *Bacillus thuringiensis*-derived gene sequences (McCown et al. 1991; Wang et al. 1996). In the present study we have used *Agrobacterium*-mediated transformation to introduce a novel *Arabidopsis thaliana* cysteine proteinase inhibitor (*Atcys*)-coding sequence in a commercial *P. alba* variety, in order to improve plant protection from insect damage. We demonstrate that the *Atcys* gene sequence is expressed in selected transgenic poplar lines and that its expression confers resistance to *C. populi* larval attack.

Materials and methods

Plant material

P. alba L. (cv. Villafranca) was obtained by controlled crossing in 1954 at the Poplar Research Institute (Casale Monferrato, Italy) and registered for commercial use in Italy and Hungary. Aseptic shoot cultures of Villafranca were maintained and propagated *in vitro* as previously described (Confalonieri et al. 2000).

Agrobacterium strain and vector plasmid

A. tumefaciens strain EHA105 containing the hypervirulent disarmed plasmid pTiBoS42 (Hood et al. 1993) was used to transform internodal stem segments. *Atcys* was isolated from *A. thaliana* (GenBank AJ000110), and was found to possess a strong cysteine proteinase inhibitory activity. Its molecular cloning and characterization will be described elsewhere. The pBI-Atcys vector plasmid (11 kb) contains a 380 bp fragment that was obtained by PCR amplification of an *Atcys* cDNA clone using two specific primers carrying a *XbaI* site (forward: TCTAGACTCGTGCCGCGAAAATGGCG) and a *Sac*I site (reverse: GAGCTCTCACGTGGTCTGAGAGCACAC) for directional cloning. The amplified fragment was sequenced and then introduced into the binary vector pBI121 (Clontech) under the control of CAMV 35S promoter, replacing the *uidA* coding region (Figure 1). The resulting binary plasmid (pBI-Atcys) was mobilized in the EHA105 disarmed strain by electroporating at 2500 V an *Agrobacterium* suspension culture grown overnight and washed with 10% glycerol. Bacterial cultures were grown in liquid LB medium (Sambrook et al. 1989) containing 150 mg/l kanamycin (Sigma) and 150 mg/l rifampicin (Lepetit) and diluted in liquid MS medium (Murashige and Skoog 1962) to achieve a density of OD_{550} 0.6 for plant transformation.

Transformation/regeneration procedure

Transformation and regeneration conditions were similar to those described by Confalonieri et al. (2000). Internodal stem segments were incubated for 24 h in the dark on a modified (3/4 macrosalts) MS callus induction medium (Murashige and Skoog 1962) supplemented with 1 mg/l IBA and 0.5 mg/l kinetin (CIM). Subsequently, they were immersed for 2 h in a bacterial suspension containing 200 μM acetosyringone. After this treatment, the explants were

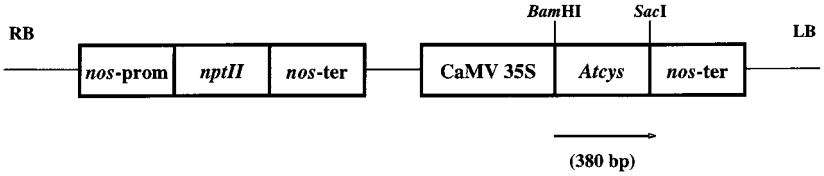

Figure 1. Schematic representation of the binary plasmid pBI-Atcys. RB, right border; *nos-prom*, nopaline synthase gene promoter; *nptII*, neomycin phosphotransferase-coding sequence; *nos*-ter, nopaline synthase gene terminator; CaMV 35S, cauliflower mosaic virus 35S promoter; *Atcys*, cysteine proteinase inhibitor coding sequence; *nos*-ter, nopaline synthase gene terminator; LB, left border.

co-cultivated for 48 h on the same medium and then placed on CIM supplemented with 300 mg/l cefotaxime, in the dark. Explants were subcultured on this medium every 10 days for the first twenty days. Subsequently, they were cut transversally in 2 to 4 pieces, transferred to light and cultured on WPM (Lloyd and McCown 1991) containing 100 mg/l kanamycin and 1 μM of TDZ, to induce shoot regeneration. After three weeks, they were subcultured on the same regeneration medium, except that TDZ was decreased to 0.01 μM. Twenty days later, growing calli were excised and cultured on selective Linsmaier and Skoog (1965) medium as modified by Chalupa (1974) containing 0.22 μM BAP. The shoots emerging from the selected calli were elongated on WPM (Lloyd and McCown 1991) in the absence of growth regulators. After 4–5 weeks, the regenerated plantlets were assayed for kanamycin resistance by testing their ability to produce roots on the same medium containing 100 mg/l kanamycin. All cultures were maintained under controlled environmental conditions (25–22 °C; 16:8 h light/dark cycle). Fifty internodal stem explants were employed for each Petri dish with two replicate plates. Transformation efficiency was defined as the number of explants which produced kanamycin-resistant plantlets relative to the total number of co-cultivated explants.

Molecular characterization

Total RNA was isolated from poplar plantlets using the small-scale procedure reported by Verwoerd et al. (1989) Aliquots containing 10 μg of total RNA extracted from fourteen different kanamycin-resistant poplar lines and from an untransformed control line were run on 1.5% w/v agarose denaturing formaldehyde gels, and subsequently stained with ethidium bromide (1 mg/ml). The separated RNAs were transferred onto nylon membranes (Appligene), according to manufacturer's instructions. Filters were hybridized as previously described (Confalonieri et al. 1998) using the purified *Bam*HI/*Sac*I fragment (380 bp) corresponding to the coding region of *Atcys* gene.

DNA extraction from untransformed and putative transgenic poplar lines was performed according to Rogers and Bendich (1988). Aliquots containing 15 μg of genomic DNA were *Eco*RI-digested (Amersham), analysed on 0.8% w/v agarose gels (Sigma) and finally transferred onto nylon membranes (Appligene). Hybridization was performed as for the northern blot analysis.

Assays of proteinase inhibition

Leaves (120 mg) from untransformed and selected transgenic plantlets were homogenized in 500 μl of 10 mM Tris pH 8.0 and centrifuged at 4 °C for 20 min at 13000 $\times$ *g*. 1 ml of 80% v/v ammonium sulphate solution was added to the supernatant, samples were incubated for 1 h in ice and then centrifuged at 4 °C for 20 min at 13000 $\times$ *g*. Subsequently, the supernatant was discarded and the pellet resuspended in 100 μl of 10 mM Tris pH 8.0. Samples were diluted to a standard protein concentration (400 μg/ml), according to Bradford (1976), and their cysteine proteinase inhibitory activity was assayed as follows. Plant protein extracts (50 μl) were mixed with 10 μl of papain solution (2 mg/ml in 50 mM phosphate buffer pH 6.8 containing 4 mM cysteine) and preincubated at 37 °C for 15 min to allow binding of inhibitor to proteinase. Subsequently, 100 μl of azoalbumin solution (10 mg/ml of 50 mM phosphate buffer pH 6.8) were added, and the samples were incubated at 37 °C for 30 min. The reaction was stopped by adding 480 μl of 10% v/v trichloroacetic acid solution. Samples were kept in ice for 15 min and then centrifuged for 3 min at 8000 $\times$ *g*. Aliquots corresponding to 500 μl of supernatant were collected and mixed with 100 μl of 3.3 M NaOH to allow staining of the undigested substrate. Inhibitory activities were calculated by measuring absorbance at 440 nm, and defined as the percentage of inhibition of the azoalbumin-hydrolysing activity of

papain. For each tested poplar line, three independent protein extracts were analysed, and the experiment was repeated two times.

Inhibitory activity of poplar protein extracts against *C. populi* midgut proteinases was determined as follows. Midguts were removed from second-instar larvae and protein extracts were obtained as previously reported (Confalonieri et al. 1998). Proteolytic activity of midgut proteins was determined with azoalbumin as substrate. The inhibition assay was carried out as described above, replacing the papain solution with a solution containing 100 μg of midgut proteins possessing an equivalent proteinase activity. Untransformed poplar plants were used as control. For each poplar line, three independent protein extracts were analysed.

Insect bioassay

An insect bioassay was performed on *P. alba* transgenic plant lines using larvae of the chrysomelid beetle (*C. populi* L.), a pest that destroys poplar and willow. Insects were collected in the field and reared in an outdoor entomological cage since 1997. Before hatching, egg batches were collected and transferred to the laboratory. For each line, 40 first-instar larvae, divided into 4 replications of 10 each, were collected by a soft, thin brush and introduced to Petri dishes. They were fed with leaves detached from plants of one control poplar line and three different transgenic lines (6EB, 14E, 21IC) expressing the *Atcys* proteinase inhibitor gene, which were grown in pot in a growth chamber. Since each replication contained larvae coming from a single egg batch, they have been considered as blocks. Petri dishes were kept in a growth chamber (24 °C, 16:8 h light/dark cycle) and leaves were replaced every day. The mortality rates of larvae before pupation and the adult emergence rates were recorded. The results were subjected to analysis of variance (randomized complete block design) for statistical evaluation of differences between control and transgenic plants. Data were transformed into arcsin $\sqrt{x}$ 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.

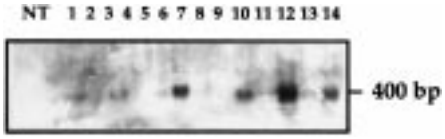

Figure 2. Northern hybridization analysis of equally loaded total RNAs extracted from leaves of fourteen kanamycin-resistant poplar lines regenerated following transformation with EHA105 pBI-Atcys *A. tumefaciens* strain, using the 380 bp fragment containing the *Atcys* gene sequence as a probe (lanes 1 to 14, respectively). Lane NT: negative control (RNA isolated from untransformed plant line).

Results

Plant transformation experiment

A total of sixteen kanamycin-resistant plant lines were regenerated from 100 cocultivated internodal explants giving a transformation frequency of 11%. Putative transgenic plants showed a normal phenotype and grew well under *in vitro* conditions. All selected kanamycin-resistant and untransformed control plantlets were micropropagated by tissue culture, transferred into pots and acclimated in a growth chamber for further evaluation.

Transgene expression and integration

Total RNAs extracted from several putative transformed poplar lines as well as the untransformed control plant were analysed by northern blot hybridization to evaluate the expression level of *Atcys* gene. The *Atcys* transcript (0.4 kb) was detected in most of the tested lines with wide variations in its steady-state level (Figure 2). The intensity of hybridization differed among the selected transgenic plant lines, even though equal amounts of total RNA were analysed. As expected, no hybridization signals were observed for the untransformed control. The strongest signal was detected for transformants 14E, 21IC, 38I and 6EB (Figure 2, lanes 7, 10, 12 and 14, respectively). However, further experiments have shown that the papain inhibitory activity in transformant 38I was lower than in the 3 other lines and this line was excluded.

Southern blot analysis performed on selected transformants using a gene-specific probe confirmed the presence of *Atcys* sequence in poplar genome. Results of this experiment are shown in Figure 3 for three different lines: several hybridization bands with different size were detected by the probe in *Eco*RI-digested DNA samples (Figure 3, lanes 2, 4 and 6) thus suggesting the presence of multiple copies of the transgene in the poplar genome. Stable integration was

Table 1. Results of a bioassay carried out on *Chrysomela populi* larvae fed with leaves of three different Atcys transgenic *Populus alba* lines.

Poplar line	Larval mortality (%)				Adult emergence
	2 days	4 days	8 days	16 days	(%)
Control	7.5	$10.0^{b}*$	$15.0^{c}*$	$17.5^{b}*$	$55.0^{b}*$
6EB	35.0	87.5^{a}	97.5^{a}	100.0^{a}	0.0^{a}
14E	15.0	35.0^{b}	40.0^{b}	77.5^{a}	7.5^{a}
21IC	12.5	65.0^{b}	90.0^{a}	100.0^{a}	0.0^{a}
F	1.84**	10.68**	17.04**	14.13**	13.14**
(df)	(3)	(3)	(3)	(3)	(3)
Probability	0.2104	0.0028	0.0006	0.0011	0.0014
Pooled SE	14.12	15.73	16.12	18.24	12.68

*Means with the same letter are not significantly different at $P < 0.05$ by the Student-Newman-Keuls multiple range test.
**Data are arcsin$\sqrt{x}$ transformed before analysis of variance.

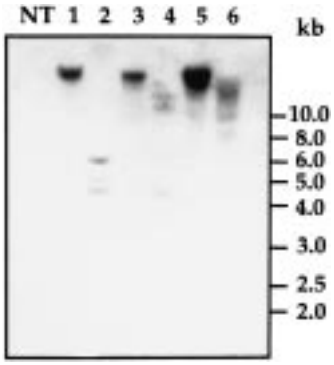

Figure 3. Results of Southern blot hybridization analysis performed on different putative transgenic poplar lines obtained following transformation with EHA105 pBI-Atcys *A. tumefaciens* strain. The 380 bp *Bam*HI/*Sac*I fragment containing the coding sequence of *Atcys* gene was used as probe. Lanes 1, 3 and 5: undigested DNAs from 14E, 21IC and 6EB poplar lines, respectively; lanes 2, 4 and 6: *Eco*RI-digested DNAs from the same poplar lines; NT: negative control (DNA isolated from untransformed poplar line).

confirmed by hybridization signals localized in the undigested DNA fraction (Figure 3, lanes 1, 3 and 5). No signal was detected using DNA extracted from untransformed control plant.

Proteinase inhibitor expression

Protein extracts from selected *Atcys* transgenic poplar lines were analysed to measure the ability of Atcys proteinase inhibitor to block *in vitro* cysteine proteinase activity. Plant extracts from 6EB, 14E and 21IC transgenic lines caused 77.5%, 53.7% and 83.7% average reduction in the papain activity, respectively. In contrast, no inhibitory effects on cysteine proteinases were recorded using control poplar plants.

To examine whether *Atcys* expression in transgenic poplars could affect *in vitro* activity of digestive proteinases from *C. populi* larvae, we determined the inhibitory activity of poplar protein extracts against a crude midgut protein preparation. With protein extracts from 6EB and 21IC transgenic lines, we observed an average decrease of 70% in midgut-derived proteinase activity. In comparison, no inhibitory activity was detected using untransformed poplar plants. These results indicated that *Atcys* products were functionally active *in vitro* and can effectively block *C. populi* midgut proteinases.

Insect bioassay performed on selected transgenic plant lines

The results of the bioassay carried out on *C. populi* larvae are listed in Table 1. Larval mortality was very low in the control line and, on the contrary, very relevant in the three selected transformed poplar lines. The transgenic line 6EB proved to be the most effective one causing significantly ($P < 0.05$) higher mortality (according to the Student-Newman-Keuls multiple range test) at day 4 from the beginning of the test, followed by line 21IC, which reached the same efficacy at day 8 and by line 14E, which did not significantly differ with respect to larval mortality from the previous ones at day 16. We did not measure the effect of transgenic poplars on larval development. However, we observed that larvae fed on all transgenic lines displayed a retarded development. Adult emergence rate was fairly normal in the control line, and significantly higher compared to transgenic lines, where it was nil or close to 0. At visual inspection, the consumed leaf area ap-

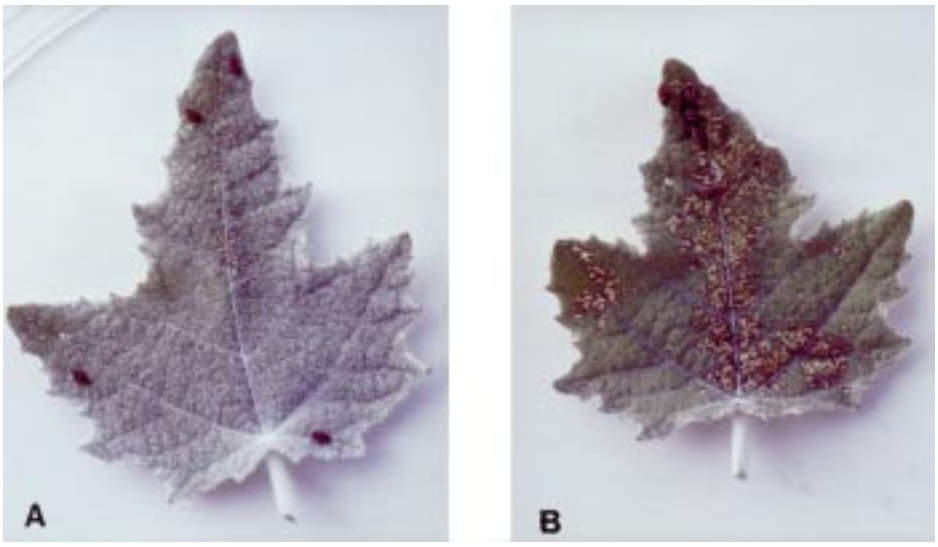

Figure 4. Comparison of resistance levels against chrysomelid beetle *(Chrysomela populi* L.) larvae between transgenic (A) and control (B) poplar lines at day 3 from the beginning of the test. Control leaf exhibited extensive damage while leaf from genetically modified plants showed little or no damage.

peared to be much larger in the control poplar line than in the three remaining transgenic lines; moreover, it was negligible in transgenic line 6EB (Figure 4), so that a really interesting protection level to pest damage can be foreseen.

Discussion

Genes of agronomic importance, such as those that confer resistance to insect pests, diseases and environmental stress, have been isolated from plants as well as from other organisms (Klopfenstein et al. 1997). More recently, established efficient regeneration/transformation systems for several poplar species (Jouanin et al. 1993) have made it possible to test the general usefulness of these genes in protecting poplars. Transgenic poplar plants with important agronomical traits have been produced by introducing several foreign genes (Klopfenstein et al. 1997). In this report we introduced a novel cysteine proteinase inhibitor *(Atcys)* gene from a dicot plant, *A. thaliana*, into an elite white poplar cultivar. Sixteen independent kanamycin-resistant poplar lines were regenerated with a transformation frequency of 11%. The presence and integration of the foreign DNA into the poplar genome was demonstrated by Southern blot analysis. The transcription level of *Atcys* proteinase inhibitor gene in different transformants was tested by northern blot hybridization analysis. *Atcys* was expressed from the CaMV 35S promoter, which is known to be a strong promoter in transgenic plants (Sanders et al. 1987). In our experiments, this sequence supported an *Atcys* expression level which allows the production of insect-resistant transgenic poplars. The enzyme inhibition assay revealed a remarkable reduction in the proteolytic activity of papain, depending on the transgenic poplar lines tested. Moreover, we have shown that *Atcys* was a very effective inhibitor of *C. populi* digestive proteinases suggesting that cysteine proteinases may account for most of the proteinase activity in *C. populi* midguts. A similar observation has been reported for the poplar leaf beetle *(Chrysomela tremulae)* by Leplé et al. (1995). and the importance of cysteine proteinases in the digestive processes of other coleopteran insects has been extensively described (Murdock et al. 1987; Wieman and Nielson 1990; Liang et al. 1991). We observed a close correspondence between the level of papain inhibition and insecticidal activity in selected *Atcys* transgenic plants. These data indicate that a relative simple *in vitro* assay could allow a rapid screening of transgenic plant lines. A similar observation was reported by Gutierrez-Campos et al. (1999) who engineered tobacco with a rice cysteine proteinase inhibitor gene against potyviruses. A high degree of insect resistance was evidenced in our bioassay. Two out of the three tested transgenic poplar lines determined 100% larval mortality 16 days after feeding, and this mortality rate was higher than ob-

served by Leplé et al. (1995). The third transgenic line showed an intermediate resistance level with a lower selection pressure on target insect. Transgenic poplar plants showing a gradation of *Atcys* gene expression may be useful for the purpose of studying modes and dynamics of the evolution of *Atcys* resistance and to develop appropriate pest management strategies.

To our knowledge this is the first report of an elite white poplar cultivar transformed with a biologically active insect resistance gene. Our study illustrates the feasibility of engineering poplar plants for resistance to *C. populi* larvae sensitive to the *A. thaliana* cysteine proteinase inhibitor. The encouraging results obtained on the insect bioassay suggest that these plants should provide a pest resistance level in the field suitable for commercial purposes. However, a field evaluation trial will be performed in order to better verify, under more realistic conditions, their efficacy in insect control.

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