Agrobacterium-mediated transformation of *Populus* × *euramericana* "Ogy" using the chimeric CaMV 35S – *pin2* gene fusion[1]

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Abstract: A hybrid poplar clone, "Ogy" (*Populus* × *euramericana*), was transformed with the potato proteinase inhibitor II (PI II) gene (*pin2*) to test the importance of serine proteinases in host–pathogen and host–insect interactions. The gene product PI II is a specific inhibitor of trypsin and chymotrypsin. Transformants were obtained using an *Agrobacterium* binary vector system with a disarmed Ti plasmid (EHA 101) and plasmid pRT104 containing *pin2* regulated by a CaMV 35S promoter and a selectable gene-encoding neomycin phosphotransferase II (NPT II). Putative transformed shoots from co-cultured leaves were selected using three different kanamycin toxicity assays. Ten plantlets, selected from 431 shoots derived from co-culture, were selected for nucleic acid and protein analysis. DNA analysis (Southern blots and PCR) and protein gel blots (Western blots) confirmed *pin2* integration and expression in transformant number TR5.

Résumé : Les auteurs ont transformé génétiquement un clone hybride de peuplier nommé « Ogy » (*Populus* × *euramericana*) avec le gène (*pin2*) codant pour l'inhibiteur de la protéinase de classe II (PI II) de la pomme de terre afin de vérifier l'importance des protéinases de la sérine dans les interactions hôtes–pathogènes et hôtes–insectes. Le produit de ce gène, PI II, est un inhibiteur spécifique à la trypsine et à la chymotrypsine. Les génotypes transformés furent obtenus à l'aide d'un système binaire du vecteur *Agrobacterium* muni d'un plasmide Ti désarmé (EHA 101) et du plasmide pRT104 contenant le gène *pin2* assujetti au contrôle régulateur du promoteur 35S du CaMV, ainsi que d'un gène de sélection codant pour la néomycine phosphotransférase II (NPT II). Des tiges présumément transformées provenant de la co-culture de feuilles furent sélectionnées à l'aide de trois tests différents de toxicité à la kanamycine. À partir de 431 tiges issues de la co-culture, 10 plantules furent choisies et soumises à l'analyse des acides nucléiques et des protéines. L'analyse de l'ADN (hybridations Southern et PCR) ainsi que les hybridations de protéines sur gel (hybridations Western) ont confirmé l'intégration du gène *pin2* et son expression dans la lignée transformée TR5.
[Traduit par la Rédaction]

Introduction

Proteinase inhibitor II (PI II) is a low molecular weight protein (monomer M_r 12 300 (Plunkett et al. 1982)) that inhibits the proteolytic activity of trypsin and chymotrypsin (Ryan 1981, 1990; Ryan and An 1988). The PI II dimer is composed of two protomers (monomers) that can vary in their molecular weight and isoelectric point (pI). The PI II dimer is capable of inhibiting 2 mol chymotrypsin/mol PI II. The inhibition of trypsin is approximately 1 mol trypsin inhibited/dimer PI II. The PI II protein is heat stable and acid soluble. The protein resists dissociation and precipitation at pH 2.2–10.6 and can be heated to 70°C for 30 min at pH 2.2 or 7.8 with minimal loss of activity (Bryant et al. 1976).

PI II has been found in solanaceous plants such as potato (*Solanum tuberosum* L.) and tomato (*Lycopersicon esculentum* Miller) (Green and Ryan 1972; Ryan 1981, 1990). PI II has been shown to reduce growth in several insect species, including *Heliothis zea* (Broddie) and *Spodoptera exiqua* (Hübner) (Broadway and Duffey 1986) and *Manduca sexta* L. (Johnson et al. 1989). It also is hypothesized that PI II could effectively reduce the vigor of fungi by depriving them of necessary amino acids (Ryan 1990). Lorito et al. (1994) demonstrated that serine proteinase inhibitors from cabbage (*Brassica oleracea* L. cv. Superpack) had an inhibitory effect on spore germination and germ tube elongation of the fungal plant pathogens *Botrytis cinerea* and *Fusarium solani* (Mart.) Sacc.. Proteinase inhibitors have reduced the activities of proteinases of *Colletotrichum lindemuthianum* (Sacc. & Magnus) (Mosolov et al. 1979) and *F. solani* (Mosolov et al. 1976). Preliminary studies from our laboratory show that *Septoria musiva* Peck (*Mycosphaerella populorum* G.E. Thompson (teleomorph)) produces proteinases that are inhibited by PI II (Sillick et al. 1989). *Septoria musiva* is able to cause loss in "Ogy" (*Populus* × *euramericana*) hybrids by causing a leaf spot and, more importantly, severe stem cankering. Ogy is also a preferred food source for both the larval and adult stages of the cottonwood leaf beetle *Chrysomela scripta* F., a major defoliator of many *Populus* selections. The digestive enzyme system of *C. scripta* is an

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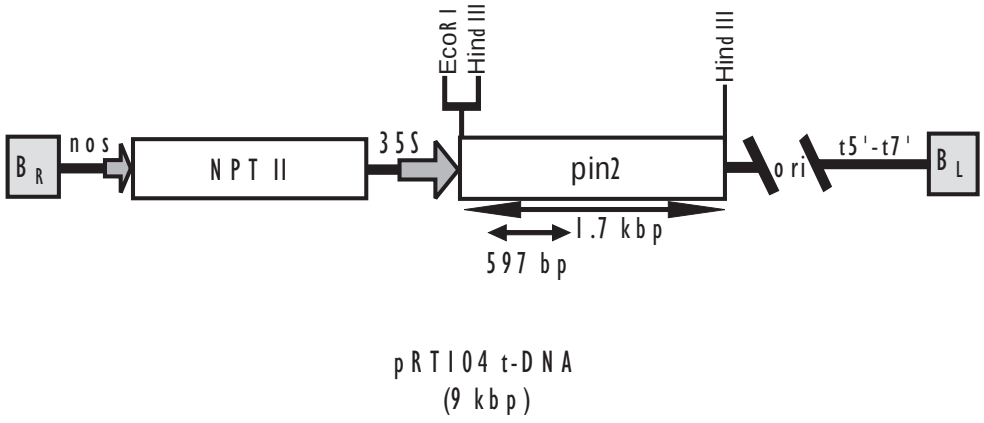
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Fig. 1. tDNA from plasmid pRT104. Relative size and location of genes *pin2*, NPT II, and their promoters in the tDNA. The omitted area (ori) represents the entire sequence of pUC13. The shaded areas labeled B_R and B_L are the right and left borders from *A. tumefaciens* tDNA. The region identified as t5′–t7′ is a bidirectional terminator. Only the restriction sites referred to in the text are shown. The *Eco*RI site mentioned in the text was used because plasmid pRT55 lacks the *Hin*dIII site found at the upstream end of the *pin2* sequence.



appropriate target for PI II. Serine proteinase activity has been observed in enzyme inhibition assays (unpublished data).

Transgenic poplars containing a chimeric fusion of the PI II gene (*pin2*) with a cauliflower mosaic virus (CaMV) 35S promoter and showing expression of PI II have been engineered to test the importance of proteinases in host–pathogen and host–insect interactions (Heuchelin et al. 1990; McNabb et al. 1990). Previous transformation of *Populus* in our laboratory has involved, in addition to constitutive promoters, the wound-inducible promoter of *pin2* and the CAT reporter gene (Klopfenstein et al. 1991). The *Populus* transformation discussed in this paper involves the hybrid clone Ogy and an *Agrobacterium* binary vector containing a disarmed Ti plasmid (EHA 101) and vector plasmid pRT104 (P. Ebert and R.W. Thornburg, unpublished). The transfer DNA (tDNA) of plasmid pRT104 contains the PI II protein-coding region and terminator of *pin2* which is regulated by the CaMV 35S promoter (Fig. 1). The tDNA also contains the neomycin phosphotransferase II (NPT II) gene, with a nopaline synthase (nos) promoter, for selectable resistance to kanamycin.

The objectives of this research project were to establish a leaf tissue regeneration system for Ogy, obtain stable transformants using the CaMV 35S – *pin2* gene construct, and develop a reliable protocol for detection of PI II in leaf tissues. The long-term goal of this research is to attain greater resistance to *S. musiva* and *C. scripta.*

Materials and methods

Culture of Ogy

Ogy is a clonal selection from the progeny of a cross of a North American *Populus deltiodes* Bartr. and the European *Populus nigra* L. Hybrids from crosses of these species are *Populus* × *euramericana* (Dode) Guinier. Ogy was selected from the breeding program of Vic Steenackers at the Poplar Research Center in Geraardsbergen, Belgium. Apical shoots of Ogy were surface sterilized with 10% ethanol and 10% chlorine bleach and placed in sterile tissue culture boxes containing MS salts (Murashige and Skoog 1962) medium (Hazleton Biological Supply, Lexena, Kans.) supplemented with B5 vitamin complex and with exogenous hormones for shoot multiplication and rooting. A clonal population for transformation was established on MSB5 shooting medium (0.2 mg naphthaleneacetic acid (NAA)/L – 0.1 mg 6-benzylaminopurine (BAP)/L). Approximately 400 shoots were rooted in MSB5 rooting medium (0.03 mg indole-3-butyric acid (IBA)/L) and grown as a sterile source of leaf material for transformation. Material was grown at 25°C under fluorescent lighting (50 $\mu E \cdot m^{-2} \cdot s^{-1}$) with a 16 h light : 8 h dark photoperiod.

Optimization of regeneration

Excised leaves of Ogy were placed on MSB5 medium containing various concentrations and ratios of BAP and NAA and incubated at 25°C under fluorescent lighting (16 h light : 8 h dark). A matrix of hormone concentrations and ratios was scored for number of shoots generated per leaf. Optimal regeneration was established at 4–6 weeks with a medium containing 0.2 mg NAA/L and 0.4 mg BAP/L. Late in the regeneration study, optimization was further enhanced by first placing the leaves on MSB5 medium containing 0.4 mg zeatin/L and, after shoot primordia were established (approximately 2 weeks), placing them on the BAP/NAA regeneration medium for shoot elongation.

Kanamycin sensitivity tests

Shoots of Ogy, 2 cm long, were placed in MSB5 rooting medium containing various concentrations (0–125 mg/L in 25-mg increments) of the antibiotic kanamycin (Sigma Chemical Co., St. Louis, Mo.). Each treatment contained 16 shoots per box with two replications per treatment. After 5 weeks, damage to shoots was assessed. A second test was conducted with concentrations ranging from 0 to 25 mg kanamycin/L.

The toxicity of kanamycin to developing shoots on whole leaves also was tested. Whole leaves were placed on the zeatin medium to induce shoot primordia and then transferred to a BAP/NAA regeneration medium containing increments of kanamycin (0–125 mg/L as above). Shoots developing on the whole leaves were observed for symptoms of kanamycin toxicity.

Co-culture with *Agrobacterium tumefaciens*

An *A. tumefaciens* strain (A281) containing a binary vector system (An 1987), consisting of plasmid pRT104 and a disarmed EHA 101 helper plasmid, was grown in MGL medium according to the protocol of Klopfenstein et al. (1991). Sterile excised leaves were wounded with forceps and placed in a MSB5 solution containing 50 μM acetosyringone (3′,5′-dimethoxy-4′-hydroxyacetophenone) and 1.0% (v/v) of the genetically engineered *A. tumefaciens* strain in MGL ($OD_{550} = 0.5$). The leaves were incubated for 48 h in the dark at 26°C with occasional gentle agitation. Leaves were rinsed repeatedly with distilled water followed by rinses containing cefotaxime (105 mg/L)

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(Hoechst-Roussel, Sommerville, N.J.) and carbenicillin (210 mg/L) (Roerig Pfizer, New York) and placed on regeneration medium containing the above antibiotics at the same concentrations.

Selection of putative transformants

First selection
After the formation of shoot primordia, the co-cultured leaves were placed on selective regeneration medium containing 50 mg kanamycin/L. After 4 weeks on the antibiotic medium, many emerging shoots were dead or severely chlorotic. Those that had remained green and vigorous were excised and placed in the second selection medium.

Second selection
Shoots 2 cm long were inserted 1 cm in MSB5 rooting medium containing 15 mg kanamycin/L. After 4 weeks, shoots were evaluated for root formation. Those producing roots were removed from the medium and were cut again to remove the rooting end of the shoot. These shoots were advanced to the third selection.

Third selection
The shoots that rooted in the second selection were placed on a MSB5 rooting medium containing 25 mg kanamycin/L. The shoots were grown on this medium for 4 weeks and evaluated for root production. Shoots that once again rooted on this higher concentration of kanamycin were removed from the medium with roots intact. These rooted shoots were washed with MSB5 solution containing cefotaxime and carbenicillin and placed in glass jars containing sterile soil mix at saturated humidity.

Removing from sterile culture
After the putative transformants had established strong root systems in the glass jars, the trees were gradually acclimated to humidities less than saturation. After 2 weeks of acclimation the trees were transplanted to 10.2 cm diameter pots and placed in an environmentally controlled growth chamber (Conviron E 15 Conviron Products of America, Pembina, N.D.). The trees were acclimated to higher light levels and air temperatures. After acclimation, the trees were grown in a glasshouse under conditions that simulated an Iowa summer (approximately 2000 $\mu E/m^2$ maximum, 15 h light : 9 h dark, 30°C, and 80% relative humidity).

DNA extraction and purification
Leaf material from Ogy was ground in liquid nitrogen and immediately placed in 60°C 2× CTAB buffer (Rogers and Bendich 1995). The mixture was heated for 1 h at the same temperature and extracted with a chloroform – isoamyl alcohol mixture (24:1). The material was separated into two phases by centrifugation at $1600 \times g$, the aqueous phase being retained and precipitated with two-thirds volume cold isopropanol. Flocculent DNA was precipitated by centrifugation at $500 \times g$. The DNA pellet was washed and centrifuged twice with wash buffer (76% EtOH and 20 mM ammonium acetate) to remove contaminants. The final pellet was resuspended in TE buffer and digested with RNase A (10 μg/mL) for 30 min at 37°C. After RNA digestion, a phenol–chloroform extraction was conducted twice followed by a chloroform – isoamyl alcohol extraction to remove protein contaminants. The DNA in the aqueous phase was precipitated by adding 0.4 volume of polyethylene glycol (PEG) solution (30% PEG 8000, 1.6 M NaCl). After 2 h of incubation at 4°C, the solution was centrifuged at $10\,000 \times g$ for 10 min. The pellet was washed with wash buffer, air dried, and resuspended in TE buffer for purity analysis.

Nucleic acid probe isolation
The probe sequence for PI II was obtained from the plasmid pRT55 (P. Ebert and R.W. Thornburg, unpublished) as described in Klopfenstein et al. (1991). The plasmid contained the PI II coding sequence and the termination sequence for the *pin2* gene. The plasmid was obtained from its bacterial host using an alkaline lysis method (Davis et al. 1986). The bacteria were grown in LB medium to an optical density of 0.8 (600 nm), chloramphenicol added (170 mg/L) to amplify the copy number of the plasmid, incubated at 37°C overnight on an orbital shaker, and extracted according to protocol. The plasmid was isolated by standard plasmid purification protocol (Davis et al. 1986). The resulting plasmid pellet was further purified with a pZ523 column (5 Prime → 3 Prime, Inc., Boulder, Co.) according to product protocol.

The purified plasmid pRT55 (4.4 kilobase pairs (kbp)) was cut with *Hin*dIII and *Eco*RI (Promega, Madison, Wis.) to yield the 1.7-kbp fragment that contained the *pin2* coding sequence and terminator (Fig. 1). The fragment was separated from the remaining plasmid sequences on a 1% agarose gel in TAE, excised from the gel, and electroeluted in dialysis tubing to obtain the DNA probe fragment.

Southern blot analysis
Purified DNA from Ogy and the transgenics was cut with the *Hin*dIII restriction enzyme, ethanol precipitated, and submitted to standard Southern blot analysis (Southern 1975). The DNA was blotted to a nylon membrane (Gene Screen Plus, New England Nuclear, Boston, Mass.) and prehybridized for 4 h at 42°C in a hybridization mixture (50% formamide, 35% 10× SSPE, 1% SDS, 5% salmon sperm DNA (10 mg/mL), 5% BSA (100 mg/mL)). The *pin2* probe fragment was labeled with α-[^{32}P]dCTP (3000 Ci/mmol, 1 Ci = 37 GBq) using a random priming reaction with the Klenow fragment (Sambrook et al. 1989). Hybridization was allowed to proceed overnight at 42°C with gentle agitation.

The membrane was rinsed three times in 2× SSC at 25°C, twice in 0.5× SSC at 50°C, twice in 0.2× SSC at 50°C, and once in 0.2× SSC at 60°C (all rinses being 15 min and containing 0.1% SDS). The membrane was placed on autoradiography film (X-Omat AR, Eastman Kodak Co., Rochester, N.Y.) with a calcium tungstate intensifier screen for 72 h at –70°C. The film was developed using standard photodevelopment technique.

Polymerase chain reaction (PCR) analysis of genomic DNA
DNA was extracted from fresh leaf tissue of untransformed control Ogy, transformant TR5, and plasmid pRT55 as decribed in the above sections. PCR analysis was conducted on the three DNA samples (templates) and a negative control that contained no template DNA. The two primers for the PCR reaction relate to positions 301–321 and 876–897 of the *pin2* gene (Thornburg et al. 1987). Amplification of the *pin2* DNA between these primers should result in a 597-bp fragment (Fig. 1). PCR reaction mixtures (50 μL) contained 50 mM KCl, 10 mM Tris–HCl (pH 9.0), 1.5 mM $MgCl_2$, 200 μM of each dNTP, 1 μM of each primer, 2.5 units of *Taq* DNA polymerase (Promega Corporation, Madison, Wis.), and 20 ng of template DNA. A MJ Research PTC-100 thermal cycler was used to run the amplification reactions. After a 60-s preheat at 95°C, 25 cycles consisting of 94°C for 60 s, 60°C for 40 s, and 72°C for 90 s were run to amplify the *pin2* sequence. The products of the PCR reactions and a 100-bp ladder were electrophoresed on a 1.4% agarose gel and stained with ethidium bromide (Sambrook et al. 1989). The gel was placed on a UV light table, and images of the PCR products were obtained with a frame grabber.

Production of antibodies to PI II
Purified PI II was purchased from Calbiochem, San Diego, Calif. New Zealand white rabbits were injected intermuscularly with PI II to elicit antibody production. Both the native and denatured forms of PI II were used as immunogens. After 4 weeks, serum was obtained and purified using a Protein A column (Bio-Rad Laboratories, Richmond, Calif.) to isolate the IgG fraction. Precipitation tests and Ochterlony double diffusion tests were run with PI II to determine the quality and cross-reactivity of the antibodies. The polyclonal IgG fraction was further purified by affinity chromatography. The antibody

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preparation was run through an Affi-gel 10 column (Bio-Rad Laboratories, Richmond, Calif.) containing proteins isolated from leaves of Ogy. Antibody that did not bind to the column was concentrated and run though an Affi-gel column containing purified PI II. Antibody that bound to the column was eluted, concentrated, and quantified.

Purification of PI II class proteins from leaves

Protein extracts from the leaves of Ogy were partially purified to obtain a group of proteins that had similar characteristics to PI II (acid solubility, heat stability, and small molecular weight). The partial purification was a modification of the PI II purification technique (Bryant et al. 1976), providing a crude inhibitor fraction. Leaves were collected from the putatively transformed trees, petiole and midrib removed, and ground with a mortar and pestle in liquid nitrogen. The frozen leaf powder was incubated in extraction buffer (0.1 M Tris–HCl, pH 7.8, 0.5 M sucrose, 0.1% ascorbic acid, 0.1% cysteine HCl, 1.0% PVP-10, 0.01 M EDTA) overnight at 4°C. The suspensions were centrifuged at $1000 \times g$ for 15 min to remove unsuspended material. The supernatant was acidified with 10 N HCl to pH 3, incubated for 1 h at 23°C, and centrifuged at $12\,000 \times g$ for 20 min to remove the acid-insoluble proteins. The acid-soluble proteins in the supernatant were then precipitated by adding $(NH_4)_2SO_4$ until the solution contained 70% of the salt. After centrifugation at $12\,000 \times g$ for 20 min, the pellet was resuspended in distilled water and heated to 80°C for 10 min to denature the heat-sensitive proteins in the mixture. The resulting turbid solution was centrifuged at $12\,000 \times g$ for 20 min to remove unsuspended material and filtered with Whatman No. 1 filter paper (Whatman International Limited, Kent, England) to remove denatured proteins that had remained suspended after centrifugation. The solution was dialyzed for 48 h against two changes of ddH_2O in Spectra/Por membranes (Spectrum Medical Industries, Inc., Houston, Tex.) with a molecular weight cutoff of 3.5 kDa. The dialyzed solution was lyophilized to obtain a partially purified protein mixture (crude inhibitor) containing PI II and any other proteins that were heat stable and acid soluble. This protein mixture was hydrated, quantified (Bradford 1976), and used in Western and colorimeteric enzyme inhibition assays.

Western blot analysis

Partially purified PI II protein (crude inhibitor) extracts from Ogy and the transgenics were prepared for electrophoresis (denatured at 100°C in SDS loading buffer containing β-mercaptoethanol) according to the protocol of Laemmli (1970), as well as two modifications of the protocol. One preparation was not heat denatured, and the other contained no β-mercaptoethanol in the loading buffer. All samples were run on a 5–22% SDS–PAGE gradient gel for 30 h at 40 V. The gel was destained in 50% methanol and 10% acetic acid to remove the tracking dye, stained with 0.05% Coomassie brilliant blue R-250 (Bio-Rad Laboratories, Richmond, Calif.) in fresh destaining solution, destained to visualize the molecular weight markers, and electroblotted onto a nitrocellulose membrane (MSI, Westboro, Mass.).

Immunodetection of the PI II proteins was accomplished by blocking the membrane with blotto containing ovalbumin (5% nonfat dry milk and 1% ovalbumin in PBS containing 0.5% Tween 20) overnight at 4°C and warming to 37°C for 30 min. After rinsing in PBS–Tween, the membrane was incubated with the polyclonal anti-PI II rabbit antibodies (see above) for 2 h at 37°C. The membrane was rinsed extensively, blocked with goat normal serum (product No. G-9023, Sigma Chemical Co., St. Louis, Mo.) for 1 h, rinsed, and incubated with anti-rabbit goat antibodies conjugated to alkaline phosphatase (product No. A-3678, Sigma Chemical Co., St. Louis, Mo.). After 1 h of incubation at 37°C, the membrane was rinsed exhaustively and placed in PBS.

PI II protein on the membrane was visualized using a NBT/BCIP (Sigma Chemical Co., St. Louis, Mo.) chemical detection system for the presence of alkaline phosphatase – antibody conjugates (Blake et al. 1984).

Development of a colorimeteric enzyme inhibition assay (EIA)

A colorimeteric EIA was developed based on the assay for protease activity on a casein substrate (Kunitz 1947). The EIA assay detected inhibition of proteinase activity by PI II. The enzymes under study were trypsin and chymotrypsin and the substrate was azocasein (Sigma Chemical Co., St. Louis, Mo.). The serine proteinase inhibitor PI II was supplied from extracts of a transgenic plant, or in control experiments, by addition of pure PI II.

The following were mixed in a glass test tube: 100 µL of leaf protein extract, 1 mL of 3% azocasein, 800 µL of phosphate buffer (0.1 M, pH 7.0), and 100 µL of 0.01% chymotrypsin or trypsin. The negative control tube (blank) contained no protease, and a positive control contained PI II in lieu of leaf extract. The tubes were incubated at 37°C for 30 min and 1 mL of the solution was placed in a clean tube. One millilitre of 5% trichloroacetic acid was added, contents mixed, and incubated for 20 min at 23°C. Contents were centrifuged for 10 min at $1100 \times g$. One millilitre of the supernatant was placed in a spectrophotometer tube that contained 1 mL of 2 N NaOH and 1 mL of ddH_2O. The contents were mixed and absorbance was read at 440 nm. One unit of proteolytic activity was defined as a decrease in absorbance of 0.01 at 440 nm. The experiment contained three replications and was run three times.

Results

Regeneration system

The optimal hormone concentration for inducing shoot primordia from leaves of Ogy was 0.4 mg zeatin/L in MSB5 medium. After induction by zeatin, optimal shoot generation was obtained with 0.4 mg BAP/L and 0.2 mg NAA/L. This new protocol yielded an average of six shoots per leaf, compared with an average of four shoots per leaf with the original regeneration protocol which relied on the BAP/NAA medium for induction of primordia (results not shown).

The ability of putatively transformed shoots to initiate root formation, and resist the toxic effects of kanamycin, was assessed after 5 weeks of culture in rooting medium containing the antibiotic. At 25 mg/L, rooting was completely inhibited and stunting was observed; as the concentration of kanamycin increased, growth was inhibited completely and chlorosis increased. In the second rooting sensitivity test, 15 mg kanamycin/L prevented rooting of the shoots, and stunting and chlorosis were minimal. At lower concentrations of kanamycin in rooting medium (10 mg/L), rooting was initiated on a few shoots. The concentration of 15 mg kanamycin/L was chosen because of its ability to prevent rooting and its low toxicity to the developing shoot.

Kanamycin sensitivity of shoots developing on whole leaves was tested. A concentration of 25 mg kanamycin/L in regeneration medium did not inhibit shoot formation, but at 50 mg/L, there was a marked reduction in shoots generated.

Selection for NPT II expression of co-cultured material

Of 431 shoots developing from co-cultured leaves, 144 remained green after the leaves were incubated for 4 weeks on medium containing 50 mg kanamycin/L (Table 1). Rooting of shoots on rooting medium containing 15 mg kanamycin/L, and later 25 mg kanamycin/L, reduced the number of shoots to 13 and 10, respectively. The three-part selection protocol greatly reduced the number of putative transformants to screen for *pin2* integration and expression. Previous studies with less rigorous selection protocols resulted in large numbers of

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Table 1. Effect of the kanamycin selection process on the number of putatively transformed shoots.

Number of leaf explants co-cultured	Shoots derived	Selection 1 (leaf and shoot), 50 mg/L	Percentage of shoots from first selection with kanamycin tolerance	Selection 2 (rooting), 15 mg/L	Selection 3 (rooting), 25 mg/L
130	95	43	45	5	3
110	87	19	22	1	1
100	109	38	35	2	2
110	78	18	23	3	3
100	62	26	42	2	1

Fig. 2. Southern blot analysis of genomic DNA from transgenic TR5 and control Ogy. Hybridization with the *pin2* DNA probe (lane 4) occurred with a 1.7-kbp fragment from the *Hin*dIII digest of TR5 (lane 1), but not with the same digest of untransformed control Ogy DNA (lane 2). Lane 3 is a λ marker (*Hin*dIII digest).

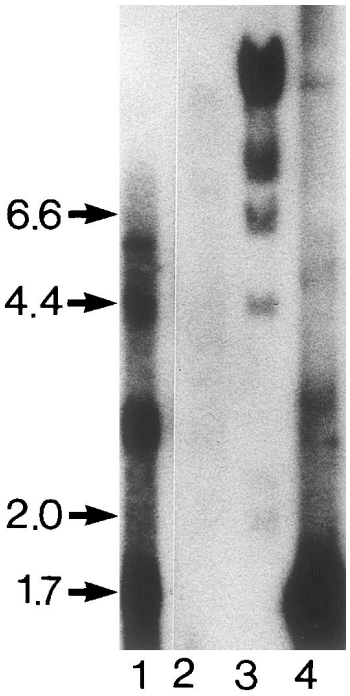
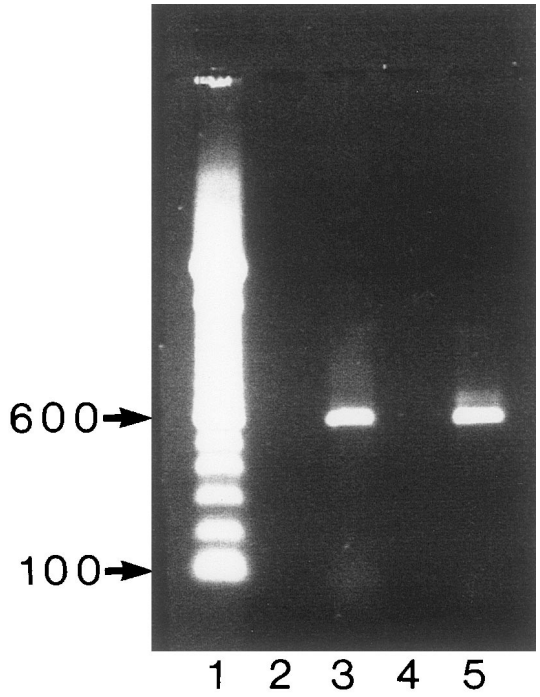


Fig. 3. PCR analysis of genomic DNA from transgenic TR5 and control Ogy. Amplification of genomic DNA by two primers that flank a 597-bp region from the *pin2* gene. Lane 1: 100-bp marker; lane 2: reaction without template DNA (negative control); lane 3: genomic DNA of TR5; lane 4: genomic DNA of untransformed Ogy; lane 5: 1.7-kbp *pin2* DNA fragment (positive control).



putative transformants to screen (Klopfenstein et al. 1991). The three-part selection protocol required more time to select for transformants, but saved considerable time and expense in later biochemical screening.

Transgenic tree No. 5 (TR5) was the most consistent transformant over all the assays for integration and expression of *pin2*. Therefore, all results and discussion pertain to TR5.

Southern analysis of genomic DNA

Hybridization with the *pin2* DNA probe occurred with a 1.7-kbp fragment from the *Hin*dIII digest of TR5 (Fig. 2, lane 1), but not with the same digest of control untransformed Ogy DNA (Fig. 2, lane 2). Pure DNA preparations were obtained with some difficulty because of large amounts of polysaccarides and other contaminating molecules that remained associated with the DNA through the extraction protocol. Several rounds of purification were often necessary to obtain high-purity DNA.

PCR analysis of genomic DNA

The PCR reaction amplified the expected 597-bp *pin2* sequence in the genomic DNA of transformant TR5 and from the DNA fragment of pRT55 (positive control). There was no visible amplification product from the sample that contained no template DNA (negative control) or the sample containing genomic DNA from the untransformed Ogy (Fig. 3).

Antibody production

The polyclonal IgG antibodies reacted strongly to native PI II (dimer) as well as to its protomers. Early in the study, Western blot analysis showed consistently high background signal in the lanes that contained extracts of control and transgenic Ogy. Affinity chromatography of the antibodies against Ogy leaf proteins provided a preparation that resulted in much lower background signal in test Westerns. Further affinity chromatography, utilizing purified PI II, resulted in a concentrated

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Fig. 4. Western blot of crude inhibitor protein extracts from transgenics and untransformed control Ogy. Lanes 1, 5, and 9: purified PI II from potato; lanes 2, 6, and 10: control Ogy; lanes 3, 7, and 11: TR5; lanes 4, 8, and 12: TR10. Lanes 1–4 were run with standard Laemmli protocol, lanes 5–8 were run without β-mercaptoethanol, and lanes 9–12 were run without heat denaturation.

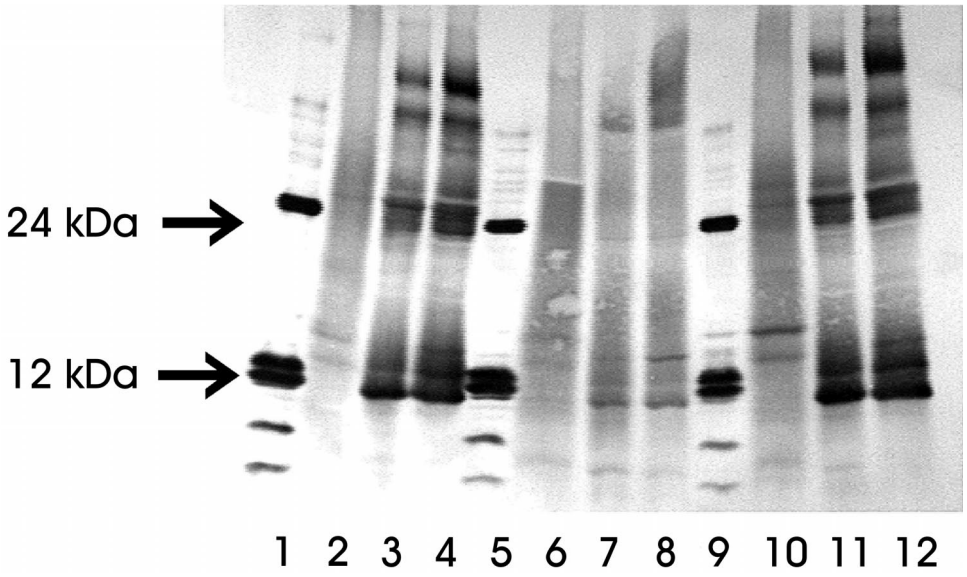
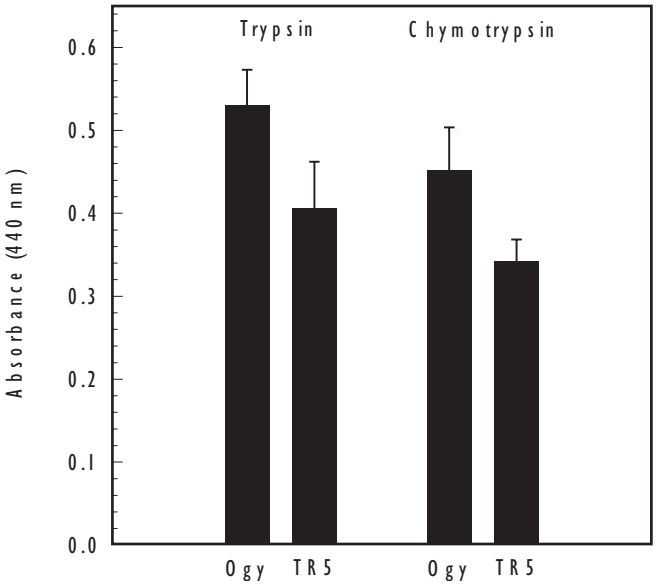


Fig. 5. Results of EIA. The effect of crude inhibitor extracts from TR5 and control Ogy on the proteolytic activity of trypsin and chymotrypsin on an azocasein substrate. Decreased absorbance indicates decreased proteolysis of the azocasein substrate by the two serine proteinases. Extracts from transgenic TR5 provided a 23.5% reduction (12.46 units) in trypsin activity and a 24.4% reduction (11.02 units) in chymotrypsin activity compared with extracts of Ogy. One unit of activity was defined as an increase in absorbance of 0.01 at 440 nm.



antibody preparation that did not bind to most native Ogy proteins, but was specific in its binding of native and denatured PI II as well as of protomers of the inhibitor.

Western blots

The presence of PI II protein was confirmed in extracts of TR5 and TR10 (Fig. 4). The PI II protein has a molecular weight of approximately 24 000 Da in its active dimer form. Protomers (monomers) of PI II are present at approximately 10 000 – 12 000 Da. Banding also was observed at molecular weights greater than 24 kDa and is most likely the result of cross-linking of monomers, which results in multimers (trimers, quatramers, etc.). The control Ogy lanes also showed banding, although at different molecular weights, caused by a native inhibitor in *Populus* that cross-reacts with anti-PI II antibody (S.A. Heuchelin, unpublished). Modifications in the Laemmli protocol of protein preparation resulted in differences in protein cross-linking and subsequent banding of multimers.

Visual observation comparisons with radial diffusion assays (Ryan 1967) show that the protein in TR5 and TR10 is being expressed at a level of approximately 50 μg/gram leaf tissue (results not shown). At this level, PI II accounts for approximately 40–50% of the proteins extracted from TR5 and TR10 with the purification protocol used in this study.

Enzyme inhibition assay

Degradation of the azocasein substrate by serine proteinases was reduced when extracted protein from transgenic TR5 was added to the assay system. The proteolytic activity of trypsin was reduced by 23.5% (12.46 units) in the presence of TR5 extract, compared with protein extracts from the untransformed control Ogy (Fig. 5). Chymotrypsin activity was reduced by 24.4% (11.02 units) when TR5 extract was compared with Ogy.

Discussion

In this study, it was evident that for each hybrid of *Populus* the regeneration and selection system must be redeveloped. Each species or clone may require different hormonal stimulation to obtain optimal regeneration and may possess varied

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tolerances to kanamycin. This transformation system was based on a previous transformation system used by our laboratory (Klopfenstein et al. 1991). Incorporating the plant hormone zeatin in the regeneration medium greatly increased the number of shoot primordia, and increased the number of putatively transformed shoots derived from the co-cultured leaves by 50%. The more stringent kanamycin selection protocol resulted in a smaller number of transgenic lines to screen, compared with the previous system used by our laboratory (data not shown).

Western blot analyses were obtained with some difficulty because of the PI II protein's unique biochemical properties. A high-density gradient gel was necessary to obtain good band separation of the PI II protein and its small molecular weight subunits. The incorporation of an initial destain of the tracking dye prior to Coomassie staining allowed for shorter destain time in the Coomassie destain process, which resulted in better stain retention by the molecular weight markers. The PI II protein has proven to be a difficult protein to work with. The formation of multimers is a real problem with this protein and its protomers. The functional PI II dimer is composed of two protomers (monomers) that can vary in their molecular weight and isoelectric point (pI). Four different protomers, A, B, C, and D, have been observed (Bryant et al. 1976). Protomer A has not been characterized because of problems in obtaining pure fractions of this protomer. The remaining three have pIs of 5.7 (B), 7.2 (C), and 8.2 (D). Any two of these protomers may combine to form one of 10 functional PI II dimers. The gel presented (Fig. 4) shows differences in banding based on sample treatment, and in each case the transgenics show novel bands compared with the control Ogy. The center group of lanes (run without β-mercaptoethanol) do not show nearly as much multimer formation. The reduction of the protein in the presence of β-mercaptoethanol appears to allow the protomers to reassociate in a plethora of combinations. We doubt that the formation of these multimers is representative of what occurs in planta, since such severe reducing conditions do not occur in the plant. In planta the protomers likely associate with one another as folded tertiary proteins to form the active dimer, not linear molecules as in the reducing gel conditions. The protomers in their reduced state may also be forming hybrids or interacting with proteins from their plant host. The large number and variety of multimers are not seen in the "pure PI II" lanes or in the control lanes, suggesting that there is an interaction between the reduced PI II protein and Ogy proteins. We have added pure PI II to control Ogy extracts and have seen the multimers form in those samples (data not shown). We also believe that the components of PI II may not migrate true to standard molecular weight size markers despite SDS treatment because of their wide-ranging pIs and strong cross-linking properties. This may explain the consistent appearance of two protomers at less than 12 kDa.

The polyclonal antibody preparation did cross-react with some proteins in the untransformed control material. We have determined that these proteins are native proteinase inhibitors of *Populus* and are currently investigating the nature of these proteins (S.A. Heuchelin, unpublished data). The double affinity purified polyclonal antibody was very useful for a variety of antibody detection methods because it was composed of a population of IgG–PI II antibodies that had a multitude of epitopes that could be recognized.

The reduction in proteolysis in the enzyme inhibition assay was not dramatic, but it may provide added resistance to pests and pathogens. Many insect pests, such as *C. scripta*, are multivoltine. If the inhibitor can reduce nutrient uptake and thus growth in the insect, there may be one less generation of larvae in a growing season. This may result in a substantial reduction of leaf area consumed and reduced vigor and numbers of overwintering adults that would start next year's infestation. The subtle inhibition may also work well for woody plant fungal pathogens that use serine proteases. Canker diseases of woody plants occur over multiple growing seasons. If the fungal canker's growth can be slowed by the proteinase inhibitor, some cankers may be halted by the tree's natural wound response.

The transformed Ogy material from this study will be a tool in understanding the host–parasite interactions of pests that use serine proteases. Previous work has shown that as little as 50 μg PI II/g leaf tissue can reduce growth severely in *M. sexta* (Johnson et al. 1989). Expression of PI II for TR5 is approximately at this level. These plants have been tested in bioassays with the insect *C. scripta* and currently are being tested with the fungus *S. musiva*.

Continued research by our group will attempt to show the effects of PI II on the host–parasite interactions of Ogy. With evidence of wound inducibility (Klopfenstein et al. 1991) and expression of an active PI II inhibitor in *Populus*, it should be possible to obtain wound-inducible expression of PI II in poplar hybrids.

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