Pergamon

Biomass and Bioenergy Vol. 12, No. 4, pp. 299–311, 1997
Published by Elsevier Science Ltd. All rights reserved
Printed in Great Britain
PII: S0961-9534(97)00004-4
0961-9534/97 $17.00 + 0.00

PROTEINASE INHIBITOR II GENE IN TRANSGENIC POPLAR: CHEMICAL AND BIOLOGICAL ASSAYS

NED B. KLOPFENSTEIN,*[1] KURT K. ALLEN,†[2] FRANCISCO J. AVILA,‡ SCOTT A. HEUCHELIN,§[3] JIMMY MARTINEZ,* RICHARD C. CARMAN,* RICHARD B. HALL,‖ ELWOOD R. HART†‖ and HAROLD S. MCNABB JR§‖

*USDA Forest Service, Rocky Mountain Forest and Range Experiment Station, National Agroforestry Center, East Campus, University of Nebraska, Lincoln, NE 68583-0822, U.S.A.
†Department of Entomology, Iowa State University, Ames, IA 50011, U.S.A.
‡Department of Plant Pathology, East Campus, University of Nebraska, Lincoln, NE 68583-0722, U.S.A.
§Department of Plant Pathology, Iowa State University, Ames, IA 50011, U.S.A.
‖Department of Forestry, Iowa State University, Ames, IA 50011, U.S.A.

Abstract—Transgenic poplar lines were developed to investigate the role of a proteinase inhibitor in pest resistance of woody plants. Using an *Agrobacterium* binary vector system, the clone 'Hansen' (*Populus alba* L. × *P. grandidentata* Michx.) was transformed with chimeric genes containing the coding region of potato proteinase inhibitor II (*PIN*2) linked to either a bacterial nopaline synthase (*nos*) or a cauliflower mosaic virus (35*S*) promoter. All transferred DNA also contained a selectable marker in the form of a *nos* promoter linked to a neomycin phosphotransferase II (*NPT II*) structural gene. The presence of the transferred *PIN*2 and *NPT II* sequences in poplar was confirmed for nine transgenic lines using polymerase chain reaction (PCR). Expression of *PIN*2 in leaves of transgenic poplar was demonstrated by enzyme-linked immunosorbent assays (ELISAs) and western blots. Two unique polypeptides from transgenic poplar, of *ca* 8 kDa and *ca* 12 kDa, indicate that *PIN*2 was translated appropriately. Resistance to the imported willow leaf beetle was tested in laboratory bioassays. The untransformed clone 'Hansen' and 11 transgenic lines were submitted to freshly hatched larvae to determine effects on pupal weight, larval development time and leaf area consumed. A significant difference from the untransformed clone in leaf area consumed was detected in one transgenic line, Tr665. Trends were indicated for several other transgenic lines for the other parameters. Published by Elsevier Science Ltd.

Keywords—*Populus*; *Agrobacterium*; proteinase inhibitor; pest resistance; genetic engineering; neomycin phosphotransferase; *Plagiodera versicolora*.

1. INTRODUCTION

Because of their desirable characteristics for biomass production, *Populus* species and hybrids are among the leading candidates for short-rotation energy and fiber forest plantations. The genus includes some of the fastest growing trees in the temperate regions and promises higher annual biomass yields than most other selections.[1] *Populus* species are also relatively easy to propagate vegetatively and can be hybridized easily intrasectionally and, to some degree, intersectionally.

Although *Populus* selections have many desirable characteristics for short-rotation forestry, there are concerns about their use. A major area of concern is with a complex of insects and pathogens that have the potential to cause problems in intensively-cultured plantations of *Populus*.[1] Such plantations are often planted to a single or limited number of clones, thereby decreasing diversity and increasing pest problems. Additionally, periodical coppicing or new plantings may maintain a high level of pest-susceptible young, succulent foliage and meristem tissue within the plantation.

Trees must possess stable pest resistance mechanisms to maintain continued growth over their long life spans. Improving host tree resistance by traditional breeding and selection is difficult because of the long generation time of trees and the rapidly changing populations of threatening pests. Molecular genetic studies designed to understand the mechanisms of host

[1]Author to whom correspondence should be addressed. Tel: 402-437-5178; Fax: 402-437-5712.
[2]Present address: USDA Forest Service, Forest Health Protection, South Dakota School of Mines and Technology, 501 E. St. Joseph, Rapid City, SD 57701, U.S.A.
[3]Present address: Department of Plant Pathology, Russell Laboratories, University of Wisconsin-Madison, 1630 Linden Drive, Madison, WI 53706, U.S.A.

resistance conferred by plant defense genes offer an alternative approach to augment programs aimed at improving pest resistance in trees.[2] Genetic engineering can contribute to improved pest resistance in trees via direct transfer of efficacious defense genes or by facilitating early selection through information gained on the roles of specific defense genes in host resistance to pests.[3-5]

When considering putative plant defense genes to transfer or select in woody plants, concerns include specificity to target pests and predicted stability of the imparted resistance trait. Defense genes encoding proteinase inhibitors are being studied because of their potential to impart host plant resistance to a wide array of insect and pathogen pests. One such gene, proteinase inhibitor II (*PIN*2), has been isolated from potato.[6] *PIN*2 encodes an inhibitor of the serine-class proteinases, trypsin and chymotrypsin.[7] These digestive enzymes are the major endopeptidases of widely distributed animal taxa (including herbivorous insects) and microorganisms (including plant pathogens).[8,9] Inhibitors of these proteinases are thought to provide foliage with anti-nutrient properties that prevent organisms from obtaining proper nourishment from the plant tissues.[10-12] This process could retard development and reproduction of insects and pathogens without causing outright mortality, thus avoiding a strong selection pressure that might cause a rapid increase in insect and pathogen resistance to the defense mechanism.[13-15] This resistance mechanism is also potentially compatible with other host defenses and other mechanisms of biological control, thereby increasing the likelihood for resistance stability.

The lack of specific genetic information for many woody plants has required the development of model systems for molecular biological studies. Current molecular biological studies are focused on *Populus* species as a model system for deciduous woody plants. Features of *Populus* spp. that have contributed to its emergence as a model system include: (1) physiological and genetic traits of *Populus* spp. are well studied; (2) tissue culture systems for *in vitro* propagation and regeneration of *Populus* are well developed;[16] (3) transformation of *Populus* spp. can be achieved with *Agrobacterium*-based systems;[17-21] (4) screening at the molecular level is facilitated by the small genome ($c = 0{\cdot}7$ pg) of *Populus* spp.;[22,23] and (6) an informative genomic map has been constructed for a hybrid poplar pedigree.[24]

Several studies have used genetic transformation of *Populus* to investigate genetic function. Such studies have been directed toward understanding transgene regulation[19,25-32] and plant growth regulator effects.[20,33-35] Other studies have focused on genes that confer herbicide resistance[18,27,36-39] or insect resistance.[40-43]

The imported willow leaf beetle, *Plagiodera versicolora* (Laicharting) (Coleoptera : Chrysomelidae), is one of several potentially important defoliators of *Populus* plantations. This insect is a multi-voltine defoliator of many species of *Salix* and *Populus*.[44] Because it can attack Leuce section poplars that are normally resistant to the cottonwood leaf beetle, *Chrysomela scripta* F., it is potentially of economic importance in plantations of aspen and white poplar.[45] Adults and larvae both feed on succulent young foliage. Such feeding can cause photosynthetic area loss and consequent biomass yield reduction, as well as destruction of the apical meristem and deformation of the tree.[45,46] In heavy infestations of first-year growth, tree mortality also may result.

To achieve the highest levels of productivity from *Populus* short-rotation plantations, pest management strategies are needed. Emphasis is being placed on multiple management approaches, especially host-plant resistance, that may serve to minimize pesticide use in these plantations. Theoretically, the product of the *PIN*2 structural gene should extend development time of the pest so that fewer generations are produced each year and the exposure of individuals to unfavorable environmental factors is increased. Also, with the decrease in food value, there should be a consequent decrease in mean fecundity, leading to reduced population growth.

In an attempt to characterize the role of proteinase inhibitors in pest resistance of woody plants, we have transformed hybrid poplar trees with gene constructs containing the *PIN*2 structural gene. This paper confirms *PIN*2 gene transfer to *Populus* and demonstrates that the gene is expressed. Bioassays were designed to screen select *Populus* lines, genetically transformed to carry the *PIN*2 structural gene, for effects on the developmental biology of the imported willow leaf beetle.

2. MATERIALS AND METHODS

2.1. *Materials*

The *Populus alba* L. × *Populus grandidentata* Michx. clone 'Hansen' served as the source material for the plant transformation. This clone is a natural hybrid that originated in southeastern Iowa.

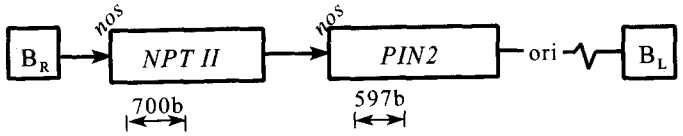
The binary vector plasmids, pRT102 and pRT104, both contain the nopaline synthase (*nos*)-neomycin phosphotransferase (*NPT II*) constructs as a selectable marker. In addition, pRT102 contains a *nos* promoter linked to a potato proteinase inhibitor II (*PIN2*) structural gene, whereas pRT104 contains a cauliflower mosaic virus 35*S* promoter linked to the *PIN2* structural gene (Fig. 1). These binary vector plasmids were incorporated into the disarmed *Agrobacterium tumefaciens* strain EHA101[47] for plant transformation.

2.2. Populus *transformation and preliminary screening*

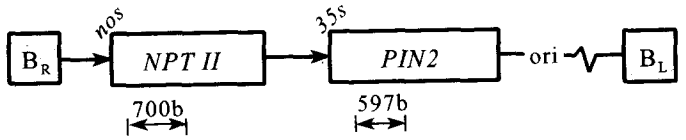
Poplar leaf cells were transformed and regenerated as described.[19] For all tissue culture manipulations of poplar, MSB5 was the basic medium; it contained MS salts,[48] B5 vitamins,[49] 3% (w/v) sucrose and 2.0 mg/l glycine. For solid media, 0.8% (w/v) agar (Difco-Bacto) was added. Hormones were added and pH was adjusted to 5.6 before autoclaving. To limit growth of *A. tumefaciens*, 500 mg/l carbenicillin and 250 mg/l cefotaxime were added to the culture media. Antibiotics were filter-sterilized and incorporated into media after autoclaving.

Leaves were excised from *in vitro* cultured plantlets and co-cultivated with the binary vector—containing *A. tumefaciens* for 2 days in liquid shoot regeneration medium containing 1.0 mg/l 6-benzylaminopurine and 0.1 mg/l 1-naphthaleneacetic acid. Following sterile rinses, co-cultivated leaves were cultured for 10 days on non-selective solid medium containing shoot regeneration hormones and antibiotics to control *A. tumefaciens*. Initial selection for transformant shoots was performed on selective shoot regeneration medium containing 40 mg/l kanamycin sulfate. Shoots forming from separate loci on the selective medium were designated as independent transformants with an individual number, and later were subjected to a secondary selection for root formation on selective rooting medium containing 0.2 mg/l indole-3-butyric acid (IBA) and 20 mg/l kanamycin sulfate. Putative transformants that passed both kanamycin screens were propagated and established as potted plantlets for greenhouse growth.

A total of 25 lines of 'Hansen' putatively transformed with pRT102 (*nos–PIN2*) and 69 lines putatively transformed with pRT104 (*35S–PIN2*) were established at the greenhouse.



pRT102 T-DNA (9kb)



pRT104 T-DNA (9kb)

Fig. 1. T-DNA maps of pRT102 and pRT104. *nos*, bacterial nopaline synthase promoter; 35*S*, cauliflower mosaic virus 35*S* promoter; *NPT II*, neomycin phosphotransferase; *PIN2*, potato proteinase inhibitor II structural gene; ori, origin of replication; B_r and B_l, border right and border left. PCR target sites are denoted between bracketed arrows.

An NPT II ELISA kit (5 Prime to 3 Prime Inc, West Chester, PA) was used to screen putative transformants further, following the protocol suggested by the manufacturer. For subsequent studies of foreign gene insertion and expression, transgenic poplar lines were selected that exhibited *NPT II* expression in leaves at the Leaf Plastochron Index (LPI)[50] 9 to 23 positions.

2.3. *Polymerase chain reaction*

Total poplar DNA was isolated from fresh leaf tissue using the CTAB method of Rogers and Bendich.[51] Based on the sequence of *PIN2*,[6] the following primers were selected: 5′-CCATCATGGATGTTCACAAGG-3′ (primer 1) and 5′-GACAAGTCTAGGGTCACATTGC-3′ (primer 2). The respective position of these primers in the *PIN2* gene is at nucleotides 301–321 and 876–897, and a 597-base pair (bp) amplified fragment is expected. An additional primer set was synthesized to identify the *NPT II* gene and consisted of the following: 5′-GAGGCTATTCGGCTATGACTG-3′ (primer 1) and 5′-ATCGGGAGCGGCGATACCGTA-3′ (primer 2).[52] The respective position of these primers in the *NPT II* gene is at nucleotides 201–222 and 879–900, and a 700 bp amplification product is expected.

Polymerase chain reactions (PCR) were conducted as 50 µl reaction mixtures. Reaction mixtures contained 10 mM Tris–HCl, pH 8.3; 50 mM KCl; 3.0 mM $MgCl_2$; 0.01% (w/v) gelatin; 200 µM of each dNTP; 2 µM of each primer; 2.5 units AmpliTaq^R polymerase (Perkin Elmer Cetus, Norwalk, CT); and 200 ng of poplar template DNA or 2 ng pRT102 plasmid template DNA. A negative control that contained no template DNA in the reaction mixture was included in each PCR run. This negative control demonstrates that the PCR reagents were free of contaminating template DNA. AmpliWax^R PCR gems (Perkin Elmer Cetus) were used as a vapor barrier to prevent evaporation from the reaction mixture. After preheating at 95°C for 1 min, 30 PCR cycles were performed using 94°C for 60 s, 55°C for 60 s, and 72°C for 90 s to amplify *PIN2* sequences or 94°C for 60 s, 64°C for 60 s, and 72°C for 90 s to amplify *NPT II* sequences. A Perkin Elmer Thermal Cycler 480 was used for all PCR temperature cycling.

PCR products were electrophoresed on a 1.6% (w/v) agarose gel and visualized with UV light after ethidium bromide staining.[53]

2.4. *Enzyme-linked immunosorbent assays*

Polyclonal antibodies were produced in rabbit against purified potato proteinase inhibitor II (PIN2) protein (Calbiochem, San Diego, CA). After differential ammonium sulfate precipitation,[54] antibodies were affinity purified with an Affi-Gel^R 10 column (Bio-Rad Laboratories Inc, Hercules, CA). To prepare labeled antibodies, alkaline phosphatase (AP) was coupled to antibodies using glutaraldehyde.[54]

Soluble proteins were extracted from leaves at the LPI 3–13 positions on greenhouse-grown plants that were approximately 2 months old and 2 m tall. Extraction methods were based on those developed by Bryant *et al.*[55] Tissues were homogenized in liquid N_2 and suspended in 0.7% (w/v) sodium dithionite with 10% (w/v) polyvinylpolypyrrolidone (PVPP). Leaf protein extracts were filtered through cheesecloth and centrifuged to remove cellular debris and PVPP, acidified to pH 3.0, and centrifuged to remove acid insoluble proteins. Soluble proteins were precipitated in 70% (w/v) ammonium sulfate, resuspended in distilled H_2O (dH_2O), heated to 80°C for 10 min, filtered through Whatman^R No.1 filter paper, dialyzed against dH_2O for 2 days, refiltered, lyophilized and stored at − 20°C. Protein extracts were resuspended in phosphate buffered saline (PBS: 136.8 mM NaCl, 2.7 mM KCl, 10.1 mM Na_2KPO_4, and 1.7 mM KH_2PO_4) at pH 7.2 and adjusted to a protein concentration of 0.5 mg/ml as determined by the method of Bradford.[56]

Each well of Immulon^{R}4 flat bottom, immunoassay plates (Dynatech Laboratories, Chantilly, VA) was coated with 4 µg affinity purified, polyclonal antibodies against *PIN2* protein in 200 ml PBS for approximately 6 h at room temperature (RT). Following six rinses in PBS, wells were blocked with 300 µl PBS + 5% (w/v) non-fat dry milk for approximately 8 h at RT, and subsequently rinsed three times with PBS + 0.05% (v/v) Tween 20 and three times with PBS. Standard curves were prepared in PBS and in untransformed (control) plant extracts in PBS. To each well, 200 µl of sample or standard curve dilution was added and incubated at 37°C for 3 h before washing three times in Tris-buffered saline (TBS: 136.8 mM NaCl, 2.7 mM KCl, and 24.8 mM Tris) pH 7.5 + 0.05% (v/v) Tween 20 and three times with TBS. For labeling, 80 ng AP-labeled antibodies were added to each well in 100 µl TBS and incubated for 2 h at 37°C before

rinsing with TBS. To develop the AP reaction, each well was covered with 100 µl substrate solution containing 3.8 mM *p*-nitrophenyl phosphate, 10 mM diethanolamine pH 9.5, and 0.5 mM $MgCl_2$. After incubation at RT in the dark, the reaction was stopped with 0.1 M EDTA and absorbance was read at 405 nm.

Each sample and standard curve was repeated three times on a plate, mean values were calculated for the plate and sample PIN2 protein concentrations were calculated from the standard curve. Each plate was replicated three times, and results were analyzed statistically with plates treated as randomized blocks. Differences in PIN2 protein levels among transgenic lines were tested by analysis of variance. PIN2 protein levels of transgenic lines were compared with the zero baseline of untransformed (control) levels by individual one-sided *t*-tests using pooled residual error of whether PIN2 protein levels differed from zero.

2.5. *Western blot analysis*

SDS–polyacrylamide gel electrophoresis (PAGE) and western blot analysis were performed according to standard protocols.[54, 57] Poplar protein samples were extracted from leaves, suspended in PBS, and adjusted for protein concentration as described for ELISA. Samples were prepared for SDS–PAGE with 12 mM Tris, pH 6.8, 2% (v/v) glycerol, 4% (w/v) SDS, 1% (v/v) 2-mercaptoethanol, and 0.00025% (w/v) bromophenol blue. After boiling for 5 min, samples containing 56 µg total protein were electrophoresed on an 18% (w/v) polyacrylamide gel with a running buffer containing 5 mM Tris, 39 mM glycine, and 0.02% (w/v) SDS. Proteins were electroblotted from the gel on to nitrocellulose using a transfer buffer containing 12 mM Tris, 96 mM glycine pH 8.3, and 20% (v/v) methanol following the protocol of the transfer apparatus manufacturer (Schleicher and Schuell Inc, Keene, NH).

After drying, nitrocellulose membranes were blocked in PBS with 5% (w/v) non-fat, dry milk for 6 h at RT. Affinity purified, polyclonal rabbit antibodies against PIN2 protein were used for unlabeled primary antibody binding at a concentration of 5 µg/ml in PBS for 24 h at RT, and rinsed five times in excess PBS. The membrane was blocked in excess normal goat serum in PBS for 6 h at RT, then washed four times in PBS, followed by two TBS washes. Alkaline phosphatase conjugated, anti-rabbit goat antibodies (Sigma Chemical Co, St Louis, MO) were used as the labeled secondary antibody at a 1/200 dilution in TBS with 3% (w/v) bovine serum albumin (BSA), and incubated with the membrane for 24 h at RT. After washing five times in excess TBS, the membrane was washed in alkaline phosphatase buffer (APB) containing 100 mM NaCl, 5 mM $MgCl_2$, and 100 mM Tris at pH 9.5. For the substrate solution, 1.2 mg nitro blue tetrazolium (NBT) dissolved in 122 µl dH_2O and 3.3 mg 5-bromo-4-chloro-3-indolyl phosphate (BCIP) dissolved in 66 µl diethylformamide were added to 10 ml APB. After suitable band development on the membrane, the reaction was stopped with PBS that contained 20 mM EDTA.

2.6. *Bioassay procedures*

Stock plants of both untransformed (control) and transformed selections (Tables 2 and 3) were maintained in the Iowa State University forestry greenhouse. All plants were watered daily and fertilized weekly with water-soluble fertilizer (20-10-20) (Peters Fertilizer Products, W. R. Grace and Co, Allentown, PA). Softwood stem cuttings were taken from the stock plants to produce sets of trees for experimental use. Cuttings, approximately two internodes long, were dipped for 5–10 s in a 500 ppm IBA solution to promote rooting, then placed in saturated peat pellets in a mist bay. Misters were programmed for 16 s activity every 4 min, while the initial rooting took place. Cuttings were left in these conditions for about 2 weeks to allow for sufficient rooting. Once the cuttings were rooted, they were potted in 12.7 cm diameter plastic pots in a mixture of equal amounts of peat, perlite and vermiculite, to which Megamp (W. R. Grace and Co, Allentown, PA), a slow-release fertilizer, was added. Potted cuttings were returned to the mist bay (16 s mist every 8 min) for about 10 days. After this adjustment period, the potted cuttings were placed randomly in an isolation bay and fertilized. Experimental plants received the same water and fertilization as stock plants. These trees were allowed to grow to the 10- to 25-leaf stage (about 6 weeks) to ensure enough foliage of an acceptable physiological age, then submitted to the beetles. For these trials, leaf material was collected from sets of trees started from cuttings as a group, to ensure that all plant material was of the same relative physiological age and grown under identical conditions.

A laboratory colony of imported willow leaf beetles was started in July 1989 from a wild

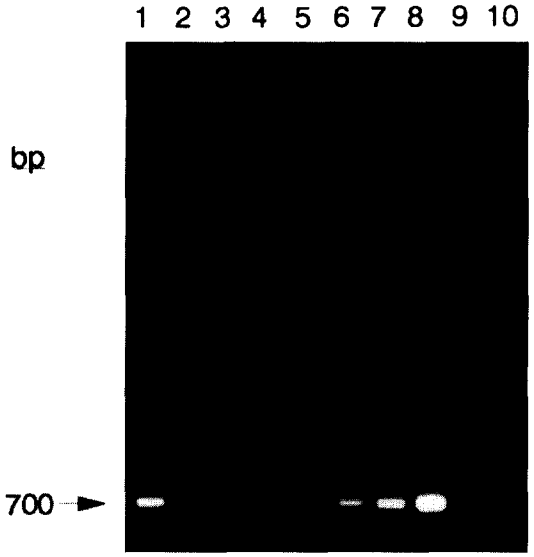


Fig. 2. PCR confirmation of *NPT II* gene in transgenic poplar. The presence of the *NPT II* gene in transgenic poplar was confirmed using polymerase chain reaction (PCR) by amplifying a target sequence within the *NPT II* selectable marker gene. Primers were selected for amplification of a 700-bp region. After temperature cycling, the PCR products were electrophoresed on a 1.6% agarose gel. Lanes 1–7 contain PCR products of template DNA from putatively transformed poplar lines as follows: lane 1, Tr555; lane 2, Tr475; lane 3, Tr338B; lane 4, Tr665; lane 5, Tr473; lane 6, Tr345; and lane 7, Tr469B. Lane 8 contains PCR products using plasmid (pRT102) DNA template that contains the *NPT II* structural gene. Lane 9 demonstrates a lack of PCR product amplification using template DNA from untransformed (control) 'Hansen' poplar. Lane 10 contains the negative control for this PCR set using PCR reagents, *Taq* polymerase, dNTPs and *NPT II* primers, but no template DNA. The negative control demonstrates that the PCR reagents were not contaminated with target DNA.

population collected near East Lansing, MI, by Dr Leah Bauer, (USDA Forest Service, North Central Forest Experiment Station, East Lansing, MI). In subsequent years, the colony was renewed each summer with field beetles from the same source. Beetles were maintained in crispers (26.4 × 19.2 × 9.5 cm) in growth chambers set to a 16 : 8 h, light : dark photoperiod, with a corresponding temperature regime of 24 : 18°C. New foliage (about 10–16 full leaves) from a variety of untransformed *Populus* clones was added to each crisper daily, with most of the old foliage being discarded. A mixture of clones was used to reduce the probability of beetle adaptation to one or a few clones. Eggs were collected daily from the crispers containing adult beetles and kept in separate containers. When the eggs hatched, the larvae were given foliage and reared through to the pupal stage. These pupae were kept and used to start the next generation.

Clonal effects of the transgenic trees were examined with larvae reared through one generation on the transformed and untransformed (control) plant material. Two series were conducted, the first series consisted of five transgenic lines and the second of six transgenic lines. The untransformed (control) clone was included in each series (Tables 2 and 3). Each line was replicated 16 times in a series.

Leaves were selected on the basis of Leaf Plastochron Index (LPI).[50] Whole excised leaves, LPI 3–7, were used for the trials. Leaves were excised from the trees at the petiole-stem junction using a razor blade. A fresh razor blade was used for each clone so that no residue was transferred from clone to clone. Immediately after leaf excision, the petiole was inserted through a septum and placed into a water-filled Aquapic (Syndicated Sales, Kokomo, IN). The leaf-Aquapic assembly was then enclosed in a plastic snapbox (12.7 × 17.8 × 3.8 cm) with a hole cut in the bottom to allow the Aquapic reservoir to protrude below the box while the leaf remained enclosed. When the cover on the snapbox was closed, a relatively isolated environment was created for the leaf. Five freshly hatched (within the previous 24 h) larvae were placed on the abaxial surface of each leaf. The larvae were allowed to feed on this first leaf for 4 days, at which time a new leaf of the same transgenic line was taken, and three surviving

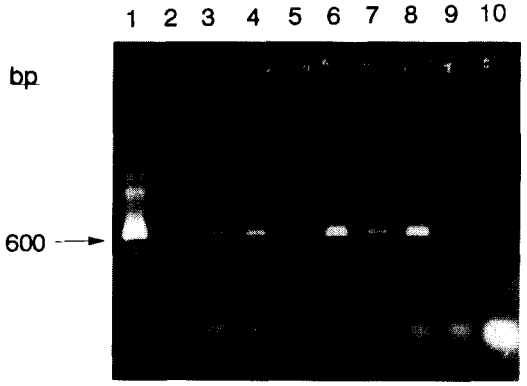


Fig. 3. PCR confirmation of *PIN2* gene in transgenic poplar. Polymerase chain reaction (PCR) was used to confirm the presence of *PIN2* sequences in separate lines of transgenic poplar. A primer set that flanks a 597-bp region within the *PIN2* structural region was used for amplification. Lane 1, plasmid pRT55 that contains *PIN2* as a positive control; lanes 2–8, seven lines of transgenic poplar, Tr338B (lane 2), Tr665 (lane 3), Tr345 (lane 4), Tr469B (lane 5), Tr555 (lane 6), Tr473 (lane 7) and Tr341 (lane 8); lane 9, amplification of template DNA from untransformed (control) 'Hansen' poplar; and lane 10: PCR negative control amplification that demonstrates that PCR reagents were not contaminated with *PIN2* sequences.

Table 1. ELISA tests of *PIN2* expression in transgenic poplar

Transgenic poplar line	Plasmid vector	N	Mean[a]		P^b
Tr555	pRT104	3	158	(86)	0.001
Tr739	pRT104	3	153	(96)	0.001
Tr475	pRT104	3	146	(94)	0.001
Tr341	pRT102	3	106	(52)	0.010
Tr338B	pRT102	3	90	(43)	0.022
Tr473	pRT104	3	87	(53)	0.025
Tr665	pRT104	3	81	(40)	0.034
Tr469B	pRT104	3	60	(30)	0.081
Tr345	pRT102	3	40	(21)	0.175

[a]ng proteinase inhibitor II per mg partially purified leaf protein.
Standard errors are parenthesized.
[b]Significance level of individual, one-sided *t*-tests of Ho: $u = o$.

Enzyme-linked immunosorbent assays (ELISAs) were used to quantify *PIN2* expression in leaves of transgenic poplar. *PIN2* encoded protein is extracted from ground leaves in sodium dithionite, acidified to pH 3, heated to 80°C for 10 min, and dialyzed.[55] Polyclonal antibodies to proteinase inhibitor II were produced in rabbit and affinity purified. Antibodies were conjugated with alkaline phosphatase, and binding was detected in the presence of *p*-nitrophenyl phosphate (pNPP) as measured at 405 nm. Readings for untransformed (control) 'Hansen' were used as a baseline.

larvae, if present, were transferred to the new leaf. The larvae were again allowed to feed for 4 days, and two survivors, if present, again moved to new foliage. Subsequently, larvae were allowed to feed until pupation on the third set of leaves. This final set of leaves was inspected daily, and pupation times were recorded. Two days after pupation, pupal weights, in milligrams, were recorded. Leaf area consumed, in square millimeters, was measured for each leaf with an area meter.

Measurements and analyses on each series included the variables of larval development time, pupal weight, and total leaf area consumed per individual larva. Total leaf area consumed was calculated on a per beetle basis by considering the amount consumed on each of the three successive leaves submitted to the beetles and the number of surviving larvae on each leaf at leaf change or on pupation.

2.7. *Bioassay analyses*

Analysis of variance tests were performed for each variable using the SAS PROC GLM procedure.[58] After analysis of variance, Tukey's multiple range tests were performed when appropriate to examine individual differences among clones.

3. RESULTS

3.1. *Polymerase chain reaction*

Examples of PCR results are presented in Figs 2 and 3. The expected 597-bp product was generated by PCR using *PIN2* primers with the *PIN2*-containing plasmid, pRT102. Bands of the same mobility were observed with genomic template DNA from nine separate lines of transgenic poplar. No other PCR products were observed from any of the reaction mixtures. Similar bands were not observed when untransformed (control) poplar DNA was added to the PCR reaction, or when template DNA was omitted from the reaction. Of the transgenic poplars that exhibited positive PCR results for *PIN2* product, three were transformed with pRT102 (Tr338B, Tr341 and Tr345) and six

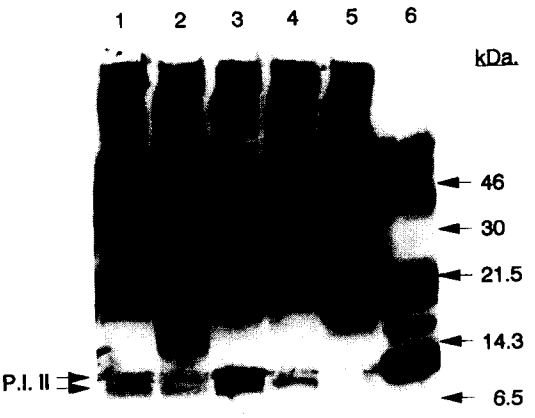


Fig. 4. Western blot demonstrating *PIN2* expression in transgenic poplars. Western blotting demonstrates that proteinase inhibitor II protein, coded by *PIN2*, is translated and processed appropriately in transgenic poplar. Proteinase inhibitor II was extracted and partially purified from transgenic poplar leaves as described for ELISA, and electrophoresed on an 18% denaturing polyacrylamide gel. Lanes 1–4, leaf protein extracts (56 μg protein) from transgenic poplar lines expressing *PIN2*; lane 5, leaf protein extract (56 μg protein) from untransformed (control) poplar; and lane 6, purified proteinase inhibitor II protein (8 μg).

Table 2. Imported willow leaf beetle mean (± SD) for pupal weight (mg), development time (days), and total leaf area consumed (mm^2) on *Populus*, Series 1

Transgenic line	Pupal weight	Development time	Leaf area consumed
'Hansen'	7.06 ± 0.91	11.38 ± 1.16	327.58 ± 117.50
Tr345	6.09 ± 1.67	11.62 ± 1.04	294.94 ± 74.03
Tr362	6.88 ± 1.82	11.20 ± 0.53	264.25 ± 70.19
Tr555	6.94 ± 0.87	11.50 ± 0.44	261.86 ± 76.76
Tr665	6.82 ± 1.85	12.00 ± 1.54	182.24 ± 84.72
Tr739	6.40 ± 0.96	11.42 ± 0.63	249.83 ± 72.53

were transformed with pRT104 (Tr469B, Tr473, Tr555, Tr665 and Tr739).

In parallel studies of the *NPT II* gene, the expected 700-bp amplification product was observed using *NPT II* primers with the *NPT II*-containing plasmid, pRT102, as a template. Amplified bands of similar size also were observed using template DNA from transgenic poplars. All transgenic poplars that possessed *PIN2* sequences also possessed sequences that corresponded to *NPT II*. No bands were observed with untransformed (control) poplar DNA serving as the template, or in the negative control reaction without template DNA.

3.2. *Enzyme-linked immunosorbent assays*

ELISA tests detected *PIN2* expression in leaves of several transgenic poplar lines (Table 1). *PIN2* expression ranged from 40–158 ng PIN2 protein/mg of partially purified leaf protein; however, results of replicated ELISA tests varied considerably. Nevertheless, a significant effect of transgenic lines on *PIN2* expression was detected. A *t*-test indicated that *PIN2* expression in seven transgenic 'Hansen' lines (Tr555, Tr739, Tr475, Tr341, Tr338B, Tr473 and Tr665) was significantly different ($\alpha = 0.05$) than untransformed (control) 'Hansen', which served as the zero baseline. Differences in *PIN2* expression among transgenic poplar lines transformed with pRT104 (*35S–PIN2*) and pRT102 (*nos–PIN2*) were not statistically significant, but the three lines with the highest *PIN2* expression were transformed with pRT104.

3.3. *Western blot analysis*

An example of western blot analysis is presented in Fig. 4. Affinity-purified, polyclonal antibodies against PIN2 protein consistently recognized multiple bands of electrophoresed PIN2 protein preparation. These bands were *ca* 8, 12 and 20 kDa in size and likely represent monomer and dimer forms of PIN2 protein.[55] Higher MW bands, possibly corresponding to multimer PIN2 protein aggregates, also were observed frequently in the lane containing PIN2 protein. Two distinct bands at *ca* 8 and 12 kDa that correspond to PIN2 protein monomers were detected in protein extracts from all transgenic poplars tested. These bands were not observed in the protein extracts from untransformed (control) poplar. Polyclonal antibodies against PIN2 protein also recognized several protein bands above *ca* 15 kDa from both transgenic and untransformed (control) poplar.

3.4. *Insect bioassays*

In series 1 (Table 2), there was no significant clonal effect on pupal weight ($F = 0.91$, $df = 5, 79$, $P < 0.4760$) or development time ($F = 1.14$, $df = 5, 76$, $P < 0.3459$). Total leaf area consumed did, however, show a significant transgenic line effect in this series ($F = 4.76$, $df = 5, 77$, $P < 0.0008$). A Tukey's studentized range test indicated that 'Hansen' differed significantly from Tr665. The table of means for this series shows some points of note. First, larvae reared on 'Hansen' consumed more total leaf area and had heavier pupal weights

Table 3. Imported willow leaf beetle mean (± SD) for pupal weight (mg), development time (days) and leaf area consumed (mm^2) on *Populus*, Series 2

Transgenic line	Pupal weight	Development time	Leaf area consumed
'Hansen'	7.60 ± 0.92	12.68 ± 1.19	273.68 ± 77.12
Tr338B	7.18 ± 2.27	12.75 ± 0.94	308.84 ± 80.50
Tr341	6.90 ± 0.96	12.50 ± 0.79	303.72 ± 80.75
Tr469B	7.52 ± 1.56	12.63 ± 1.03	340.89 ± 75.87
Tr473	7.27 ± 1.05	13.00 ± 1.32	333.76 ± 120.47
Tr475	6.89 ± 1.36	13.32 ± 1.60	283.92 ± 78.04
Tr511	6.95 ± 0.96	13.32 ± 0.80	333.52 ± 176.91

than those reared on the five other transgenic lines. Secondly, larvae reared on clone Tr665 consumed the least total leaf area and had the longest development period. Thirdly, larvae reared on clone Tr345 consumed a relatively large amount of total leaf area, but had the least pupal weight.

In series 2 (Table 3), pupal weight ($F = 0.61$, $df = 6, 84$, $P < 0.7230$), development time ($F = 1.18$, $df = 6, 83$, $P < 0.3275$), and total leaf area consumed ($F = 0.81$, $df = 6, 83$, $P < 0.5653$) were all non-significant. However, two points may be noted. First, larvae reared on 'Hansen' consumed the least total leaf area in this series, yet had the heaviest pupal weight. Secondly, larvae reared on 'Hansen' had shorter development times than on most other clones (up to 0.6 days sooner on 'Hansen'), while achieving heavier pupal weights.

4. DISCUSSION

4.1. *Transformations*

Regeneration rates of putatively transgenic poplars were similar to those previously reported.[19] Because this study focused on obtaining a series of *PIN2* expressing lines of transgenic poplar, efforts were not made to verify the lack of transferred genes in lines that did not pass various screening procedures. Thus, transformation frequencies are difficult to estimate.

PCR analysis indicates that all of the nine transgenic poplar lines examined contain both the *NPT II* and *PIN2* genes, thereby indicating that T-DNA was transferred intact by the *Agrobacterium* binary vector system. PCR provides a relatively simple and consistent method for demonstrating the transformation of poplar with foreign genes. In this study, extra steps were used to semi-purify the poplar DNA before amplification. Simplified methods that start with small leaf pieces as the source of template DNA[59] subsequently were attempted with success (data not shown). Continued development of such simplified methods for the amplification of poplar DNA will greatly simplify screening of putative transformants.

A range of *PIN2* expression is expected, based on the number of inserted genes, the insertion site or "position effect" of the foreign gene, and interacting developmental and environmental influences.[60] Differential effects on expression by *nos* and 35*S* promoters are also expected.[30] Differences in *PIN2* expression among transgenic poplar lines were noted; however, precise ELISA quantification of *PIN2* expression in transgenic poplar was hampered by marked variation both within and between replicate tests. This variation is likely due to interference related to the endogenous poplar proteins recognized by polyclonal antibodies against PIN2 protein. Such cross-reactive endogenous proteins of poplar are perhaps encoded by native genes with high homology to *PIN2*. It is presently unknown whether these native poplar proteins possess proteinase inhibitor activity; however, other workers have demonstrated that *Populus* spp. possess genes with homology to proteinase inhibitors.[61,62]

Western blotting demonstrated that *PIN2* is apparently translated appropriately in transgenic poplar. Native poplar proteins also demonstrated cross-reactivity with polyclonal antibodies that were prepared against PIN2 protein. Ambiguities caused by non-specific antibody binding could be alleviated with monoclonal antibodies that recognize PIN2 protein, but not native poplar proteins. Additional studies are needed to determine specific proteinase inhibition activity of the transgene encoded PIN2 protein, and whether the foreign PIN2 protein is correctly localized within the central vacuole of poplar cells.

Because *PIN2* is expressed in the leaves, the transgenic poplars can be used for bioassays with leaf-feeding insects and leaf-infecting pathogens. Transgenic lines of hybrid poplar that express *PIN2* were selected for further studies to characterize the role of *PIN2* in pest resistance. Initial insect resistance studies were designed to use transgenic poplar to evaluate the effects of *PIN2* on growth and development of the imported willow leaf beetle. Additionally, the role of proteinase inhibitors in resistance to diseases is ill-defined. Future studies should be directed toward evaluating spatial and temporal expression of *PIN2* in transgenic poplar, so that effects on poplar pests of the leaf, stem or root can be characterized. Additional studies are needed to monitor gene expression over time as the transgenic poplars mature. It is likely that the biological influence of introduced *PIN2* genes in *Populus* will depend on interactions with other native defense genes. Such interactions will become better understood with increased study of native defense gene systems of *Populus*.

4.2. *Bioassays*

Although few statistically significant differences occur among the results, some trends were indicated. In all instances, weights for larvae reared on untransformed (control) lines were heavier than for those reared on transgenic lines. This was consistent, despite the leaf area consumed, suggesting that the untransformed "parent" clone may have a greater nutritional value than the transgenic clones. In series 1 of the single generation tests, Tr665 had significantly less total leaf area consumed than the untransformed (control) clone. Beetles reared on this transgenic line had the longest development time in series 1, spending more than 0.5 days longer to reach pupation than those reared on untransformed 'Hansen'.

The development times remained fairly constant in both series, with the range for both being 11.2–13.3 days. This is slightly less than the average time spent as larvae in the field,[63,64] about 14.5 days, and the time spent as larvae reared in an insectary, about 14.8 days.[64] This shortening of time spent as larvae is probably caused by the optimal rearing conditions in the laboratory.

In a similar study by Leplé *et al.*,[41] the proteinase activity profile of third instar, *C. tremulae* larvae was shown to contain both cysteine and serine proteinases. For that study, hybrid poplar (*Populus tremula* × *P. tremuloides*) was transformed with a gene encoding oryzacystatin OCI, a cysteine proteinase inhibitor, to produce transgenic poplars that produced OCI at relatively high levels (*ca* 2% of soluble proteins) in young leaves. After a 25-day feeding period, toxicity tests of OCI-expressing poplar leaves produced up to 43.5% mortality to *C. tremulae* larvae, compared with 4.5% mortality with the untransformed (control) poplar. In addition, adverse effects on growth and development were noted for insects reared on the transgenic poplar.[41] Although those results are not directly comparable with the present study, they further support the role of proteinase inhibitors in resistance of poplar to leaf-feeding chrysomelids.

In other previous work, transgenic tobacco has been used to examine the role of proteinase inhibitor genes in resistance to insects. Expression of an introduced cowpea trypsin inhibitor gene was correlated with a decreased survival of feeding *Heliothis virescens* (Lepidoptera: Noctuidae) larvae.[65] In addition, growth rates of feeding *Manduca sexta* (Lepidoptera: Noctuide) larvae were suppressed when fed transgenic tobacco that expressed either an introduced tomato or potato *PIN*2 gene.[66] In similar work by McManus *et al.*,[67] tobacco was transformed with a member of the *PIN*2 gene family that encodes a chymotrypsin *iso*-inhibitor. Trials with *Chrysodeixis eriosoma* (Lepidoptera: Noctuidae, green looper) demonstrated that larvae grew slower when fed leaf tissue from transgenic tobacco that was expressing *PIN*2. However, the transgenic tobacco produced no larval growth inhibition for two other insects, *Spodoptera litura* (F.) (Lepidoptera: Noctuidae) and *Thysanoplusia orichalcea* (F.) (Lepidoptera: Noctuidae). Differences in feeding assays corresponded to differences in digestive proteinases of the insect species. It was concluded that proteinase inhibitors should be evaluated as an effective strategy to provide transgenic plants with resistance to certain insects; however, a single proteinase inhibitor gene may not be universally effective against a range of insect pests.[67]

The main concerns of using proteinase inhibitors as a defense are the time required to achieve control and the possibility of pest tolerance. Tolerance is gained by changes in the pest digestive enzyme complex.[15] This change is possible because of the great diversity of proteinase activity present in insects.[68] Selection pressure would be reduced by placing the defense gene under the control of a wound-inducible promoter. Any genetic transformation to enhance resistance should be considered carefully. Trees are long lived, and the pest population will have many generations in which to evolve resistance to the new trait.[14] Insects often have several mechanisms to counteract plant defenses, so evolution can be rapid.[69]

The results of this work lead to some tentative conclusions. Some transgenic clones indicate a tendency toward resistance to this insect. This resistance is exhibited in the form of smaller pupae being produced and/or longer development times for the larvae. It is assumed that smaller pupae are formed by larvae that fed on a food source with reduced nutritional value. This process could produce adults that are less fecund, thereby producing less offspring. The larval stage is most susceptible to mortality from biotic and abiotic forces. If the duration of this stage is lengthened, the chances increase for natural suppression factors to reduce the population.

Variation in *PIN2* expression among transgenic lines is expected because of position effects, based on the site of *PIN2* integration into the poplar genome.[60] Foreign gene expression can also be influenced by plant development and the environment. The transgenic poplar lines demonstrate variable *PIN2* expression levels; however, proteinase inhibitor II levels were not determined for leaves used in insect feeding assays. Based on *PIN2* expression levels and the insect feeding bioassay data, several transgenic lines must be screened to select transgenic lines with optimal resistance levels.

Work in this system is needed in several areas. The resistance of the trees needs to be tested with intact plants under field conditions, rather than plant parts under controlled conditions. Using parts removed from the tree may limit available resources to produce this resistance protein. With whole trees available as a resource base, the plants can potentially produce more resistance product over a longer time so that effects on beetle biology are magnified. Resistance may also be increased when insects feed on less preferred leaves. Also, trees carrying resistance genes may be more effective when combined with the natural enemies and less favorable developmental conditions found in a plantation environment. Finally, work is needed to determine how these transgenic trees can be used best in a plantation environment.

Acknowledgements—The authors thank Dr Robert W. Thornburg for providing the plasmids used for the poplar transformation and information regarding assays of *PIN2* expression, Michael J. Kuhl and Jill G. Kerl for technical help in conducting ELISAs, and Rudy M. King and Mee-Sook Kim for assisting in the statistical analyses. We thank Vincent Smith for assistance with the bioassays, Rich Faltonson for guidance with plant propagation and care, and Dr Leah Bauer for the insects used in the study. Manuscript reviews were provided by Drs Carol M. Schumann and Young Woo Chun. This research was supported in part by funds from the Consortium for Plant Biotechnology Research, Inc (contract # DOE OR22072-17 to H.S.M., R.B.H. and N.B.K.), Iowa Agriculture and Home Economics Experiment Station projects 2379, 2976 and 3088 (to R.B.H., H.S.M. and E.R.H.), subcontract No. 19X-43391C with Oak Ridge National Laboratory under Martin Marietta Energy Systems Inc contract DE-AC05-840R21400 with the U.S. DOE (to R.B.H., H.S.M. and E.R.H.), cooperative research agreement No. 28-C2-637 from the USDA Forest Service (to H.S.M. and R.B.H.), and the Agricultural Biotechnology Council of Iowa State University (to H.S.M., R.B.H., E.R.H. and N.B.K.). Use of trade names in this paper does not constitute endorsement by the USDA Forest Service. Journal Paper No. J15993 of the Iowa Agriculture and Home Economics Experiment Station, Ames, IA.

REFERENCES

1. Dickmann, D. I. and Stuart, K. W., *The Culture of Poplars in Eastern North America*. Michigan State University Press, East Lansing, MI, 1983.
2. Klopfenstein, N. B., McNabb, H. S. Jr., Hart, E. R., Hall, R. B., Hanna, R. D., Heuchelin, S. A., Allen, K. K., Shi, N.-Q. and Thornburg, R. W., Transformation of *Populus* hybrids to study and improve pest resistance. *Silvae Genet.*, 1993, **42**, 86–90.
3. Ernst, S. E. and Klopfenstein, N. B., Improvement of woody plant germplasm using a combination of conventional and molecular research techniques. In *Trees Are the Answer: 42nd Annual Meeting, Forestry Committee, GPAC*. Great Plains Agricultural Council Publication 132, Colorado Springs, CO, pp. 36–40, 1990.
4. Klopfenstein, N. B. and Kerl, J. G., The potential of biotechnology in temperate agroforestry practices. *Agroforestry Systems*, 1995, **32**, 29–44.
5. Schumann, C. M. and Klopfenstein, N. B., Contributions of molecular biology to improvement of disease resistance in Great Plains tree species. In *Biological Control of Forest Pests in the Great Plains: Status and Needs—A Symposium*. Proceedings of the 44th annual meeting of the Forestry Committee, Great Plains Agricultural Council. Great Plains Agricultural Council Publication No. 145, Bismarck, ND. pp. 102–104, 1993.
6. Thornburg, R. W., An, G., Cleveland, T. E., Johnson, R. and Ryan, C. A., Wound-inducible expression of a potato inhibitor II—chloramphenicol acetyltransferase gene fusion in transgenic tobacco plants. *Proc. Natl Acad. Sci. USA*, 1987, **84**, 744–748.
7. Ryan, C. A., Protease inhibitors in plants: genes for improving defenses against insects and pathogens. *Ann. Rev. Phytopath.*, 1990, **28**, 425–449.
8. Broadway, R. M. and Duffey, S. S., Plant proteinase inhibitors: Mechanism of action and effect of the growth and digestive physiology of larval *Heliothis zea* and *Spodoptera exiqua*. *J. Insect Physiol.*, 1986, **32**, 827–833.
9. Mosolov, V. V., Loginova, M. D., Fecurkina, N. V. and Benken, I. I., The biological significance of proteinase inhibitors of plants. *Plant Sci. Letts*, 1976, **7**, 77–80.
10. Broadway, R. M., Duffy, S. S., Pearce, G. and Ryan, C. A., Plant proteinase inhibitors: A defense against herbivorous insects?. *Entomol. Exp. Appl.*, 1986, **41**, 33–38.
11. Ryan, C. A., Proteinase inhibitors. In *The Biochemistry of Plants*, Vol. 6, Academic Press, New York, pp. 351–370, 1981.
12. Wolfson, J. L., The effects of induced plant proteinase inhibitors on herbivorous insects. In *Phytochemical Induction by Herbivores*, eds D. W. Tallamy and Raupp, M. J. John Wiley and Sons, New York, pp. 223–243, 1991.
13. Gould, F., Evolutionary biology and genetically engineered crops. *BioScience*, 1988, **38**, 26–33.
14. Raffa, K. F., Genetic engineering of trees to enhance resistance to insects. *BioScience*, 1989, **39**, 524–534.
15. Thornburg, R. W., New approaches to pest resistance in trees. *AgBiotech News Inf.*, 1990, **2**, 845–849.
16. Chun, Y. W., Klopfenstein, N. B., McNabb, H. S. Jr. and Hall, R. B., Biotechnical applications in *Populus* species. *J. Korean For. Soc.*, 1988, **77**, 467–483.
17. Chun, Y. W., Klopfenstein, N. B., McNabb, H. S. Jr. and Hall, R. B., Transformation of *Populus* species by an *Agrobacterium* binary vector system. *J. Korean For. Soc.*, 1988, **77**, 199–207.
18. Fillatti, J. J., Sellmer, J., McCown, B., Haissig, B. and Comai, L., *Agrobacterium* mediated transformation and regeneration of *Populus*. *Mol. Gen. Genet.*, 1987, **206**, 192–199.

19. Klopfenstein, N. B., Shi, N.-Q., Kernan, A., McNabb, H. S. Jr., Hall, R. B., Hart, E. R. and Thornburg, R. W., Transgenic *Populus* hybrid expresses a wound-inducible potato proteinase inhibitor II–CAT gene fusion. *Can. J. For. Res.*, 1991, **21,** 1321–1328.
20. Parsons, T. J., Sinkar, V. J., Stettler, R. F., Nester, E. W. and Gordon, M. P., Transformation of poplar by *Agrobacterium tumefaciens*. *Bio/Technology*, 1986, **4,** 533–536.
21. Pythoud, F., Sinkar, V., Nester, E. W. and Gordon, M. P., Increased virulence of *Agrobacterium rhizogenes* conferred by the *vir* region of pTiBo542: Application to genetic engineering of poplar. *Bio/Technology*, 1987, **5,** 1323–1327.
22. Dhillon, S. S., Miksche, J. P. and Cecich, R. A., DNA changes in senescing leaves of *Populus deltoides*. *Plant Physiol.* (*Suppl.*), 1984, **75,** 120.
23. Parsons, T. J., Bradshaw, H. D. Jr. and Gordon, M. P., Systemic accumulation of specific mRNAs in response to wounding in poplar trees. *Proc. Natl Acad. Sci. USA*, 1989, **86,** 7895–7899.
24. Bradshaw, H. D. Jr. and Stettler, R. F., Molecular genetics of growth and development in *Populus*. IV. Mapping QTLs with large effects on growth, form, and phenology traits in a forest tree. *Genetics*, 1995, **139,** 963–973.
25. Chun, Y. W. and Klopfenstein, N. B., Organ specific expression of the *nos–NPT II* gene in transgenic hybrid poplar. *J. Korean For. Soc.*, 1995, **84,** 77–86.
26. Confalonieri, M., Balestrazzi, A. and Bisoffi, S., Genetic transformation of *Populus nigra* by *Agrobacterium tumefaciens*. *Plant Cell Reports*, 1994, **13,** 256–261.
27. De Block, M., Factors influencing the tissue and the *Agrobacterium tumefaciens*-mediated transformation of hybrid aspen and poplar clones. *Plant Physiol.*, 1990, **93,** 1110–1116.
28. Devantier, Y. A., Moffatt, B., Jones, C. and Charest, P. J., Microprojectile-mediated DNA delivery to the Salicaceae family. *Can. J. Bot.*, 1993, **71,** 1458–1466.
29. Kajita, S., Osakabe, K., Katayama, Y., Kawai, S., Matsumoto, Y., Hata, K. and Morohoshi, N., *Agrobacterium*-mediated transformation of poplar using a disarmed binary vector and the overexpression of a specific member of a family of poplar peroxidase genes in transgenic poplar cell. *Plant Sci.*, 1994, **103,** 231–239.
30. Leplé, J. C., Brasileiro, A. C. M., Michel, M. F., Delmotte, F. and Jouanin, L., Transgenic poplars: expression of chimeric genes using four different constructs. *Plant Cell Rep.*, 1992, **11,** 137–141.
31. Nilsson, O., Alden, T., Sitbon, F., Little, C. H. A., Chalupa, V., Sandberg, G. and Olsson, O., Spatial pattern of cauliflower mosaic virus 35s promoter-luciferase expression in transgenic hybrid aspen trees monitored by enzymatic assay and non-destructive imaging. *Transgenic Res.*, 1992, **1,** 209–220.
32. Tsai, C.-J., Podilla, G. K. and Chiang, V. L., *Agrobacterium*-mediated transformation of quaking aspen (*Populus tremuloides*) and regeneration of transgenic plants. *Plant Cell Rep.*, 1994, **14,** 94–97.
33. Charest, P. J., Stewart, D. and Budicky, P. L., Root induction in hybrid poplar by *Agrobacterium* genetic transformation. *Can. J. For. Res.*, 1992, **22,** 1832–1837.
34. Schwartzenberg, K. V., Doumas, P., Jouanin, L. and Pilate, G., Enhancement of the endogenous cytokinin concentration in poplar by transformation with *Agrobacterium* T-DNA gene *ipt*. *Tree Physiol.*, 1994, **14,** 27–35.
35. Wang, S.-p., Xu, Z.-h. and Wei, Z.-m., Genetic transformation of leaf explants of *Populus tomentosa*. *Acta Botanica Sinica*, 1990, **32,** 172–177.
36. Brasileiro, A. C. M., Tourneur, C., Leplé, J.-C., Combes, V. and Jouanin, L., Expression of the mutant *Arabidopsis thaliana* acetolactate synthase gene confers chlorsulfuron resistance to transgenic poplar plants. *Transgenic Res.*, 1992, **1,** 133–141.
37. Devillard, C., Transformation *in vitro* du tremble (*Populus tremula* × *Populus alba*) par *Agrobacterium rhizogenes* et regeneration de plantes tolerantes au basta. *C. R. Acad. Sci. Paris*, 1992, **314 III,** 291–298.
38. Donahue, R. A., Davis, T. D., Michler, C. H., Riemenschneider, D. E., Carter, D. R., Marquardt, P. E., Sankhla, N., Sankhla, D., Haissig, B. E. and Isebrands, J. G., Growth, photosynthesis, and herbicide tolerance of genetically modified hybrid poplar. *Can. J. For. Res.*, 1994, **24,** 2377–2383.
39. Riemenschneider, D. E. and Haissig, B. E., Producing herbicide tolerant *Populus* using genetic transformation mediated by *Agrobacterium tumefaciens* C58: A summary of recent research. In *Woody Plant Biotechnology*, ed. M. R. Ahuja. Plenum Press, New York, pp. 247–263, 1991.
40. Howe, G. T., Goldfarb, B. and Strauss, S. H., *Agrobacterium*-mediated transformation of hybrid poplar suspension cultures and regeneration of transformed plants. *Plant Cell Tissue Organ Cult.*, 1994, **36,** 59–71.
41. Leplé, J. C., Bonadé-Bottino, M., Augustin, S., Pilate, G., Dumanois Lê Tân, V., Delplanque, A., Cornu, D. and Jouanin, L., Toxicity to *Chrysomela tremulae* (Coleoptera : Chrysomelidae) of transgenic poplars expressing a cysteine proteinase inhibitor. *Molecular Breeding*, 1995, **1,** 319–328.
42. McCown, B. H., McCabe, D. E., Russell, D. R., Robison, D. J., Barton, K. A. and Raffa, K. F., Stable transformation of *Populus* and incorporation of pest resistance by electric discharge particle acceleration. *Plant Cell Rep.*, 1991, **9,** 590–594.
43. Robison, D. F., McCown, B. H. and Raffa, K. F., Responses of gypsy moth (Lepidoptera: Lymantriidae) and forest tent caterpillar (Lepidoptera: Lasiocampidae) to transgenic poplar, *Populus* spp., containing a *Bacillus thuringiensis* d-endotoxin gene. *Environ. Entomol.*, 1994, **23,** 1030–1041.
44. Raupp, M. J., The spatial distribution and seasonal abundance of the imported willow leaf beetle, *Plagiodera versicolora* Laich.: The effects of plant nutrition and defense, physical factors, and activities of competitors and predators. PhD dissertation, University of Maryland, College Park, MD, 1982.
45. Ostry, M. E., Wilson, L. F., McNabb, H. S. Jr. and Moore, L. M., A guide to insect, disease, and animal pests of poplars. *USDA Forest Service, Agriculture Handbook*, 1989, **677,** 118 pp.
46. Randall, W. K., Willow clones differ in susceptibility to cottonwood leaf beetle. In *Proceedings, 11th Conference on Southern Forest Tree Improvement*. Southern Forest Tree Improvement Committee, Atlanta, GA, pp. 108–111, 1971.
47. Hood, E. E., Helmer, G. L., Fraley, R. T. and Chilton, M.-D., The hypovirulence of *Agrobacterium tumefaciens* A281 is encoded in a region of pTiBo542 outside of T-DNA. *J. Bacteriol.*, 1986, **168,** 1291–1301.
48. Murashige, T. and Skoog, F., A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiol. Plant.*, 1962, **15,** 473–497.
49. Gamborg, O. L., Miller, R. A. and Ojama, K., Nutrient requirements of suspension cultures of soybean root cells. *Exp. Cell Res.*, 1968, **50,** 151–158.
50. Larson, P. R. and Isebrands, J. G., The plastochron index as applied to developmental studies of cottonwood. *Can. J. For. Res.*, 1971, **1,** 1–11.
51. Rogers, S. O. and Bendich, A. J., Extraction of DNA from plant tissues. In *Plant Molecular Biology Manual*, eds S. B. Gelvin and Schilperoot, R. A.. Kluwer

Academic Publishers, Boston, MA, Part A6, pp. 1–10, 1988.
52. Hamill, J. D., Rounsley, S., Spencer, A., Todd, G. and Rhodes, M. J. C., The use of the polymerase chain reaction in plant transformation studies. *Plant Cell Rep.*, 1991, **10,** 221–224.
53. Sambrook, J., Fritsch, E. F. and Maniatis, T., *Molecular Cloning*: *A Laboratory Manual*, 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989.
54. Harlow, E. and Lane, D., *Antibodies*: *A Laboratory Manual*. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1988, 726 pp.
55. Bryant, J., Green, T. R., Gurusaddaiah, T. and Ryan, C. A., Proteinase inhibitor II from potatoes: Isolation and characterization of its protomer components. *Biochemistry*, 1976, **15,** 3418–3423.
56. Bradford, M. M., A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principles of protein-dye binding. *Anal. Biochem.*, 1976, **72,** 248–254.
57. Coligan, J. E., Kruisbeek, A. M., Margulies, D. H., Shevach, E. M. and Strober, W. (eds) *Current Protocols in Immunology*. Greene Publishing Associates and Wiley-Interscience, New York, 1991.
58. SAS Institute, *SAS Users Guide*: *Statistics*, 5th edn. SAS Institute, Cary, NC, 1985.
59. Berthomieu, P. and Meyer, C., Direct amplification of plant genomic DNA from leaf and root pieces using PCR. *Plant Mol. Biol.*, 1991, **17,** 555–557.
60. Peach, C. and Velten, J., Transgene expression variability (position effect) of CAT and GUS reporter genes driven by linked divergent T-DNA promoters. *Plant Mol. Biol.*, 1991, **17,** 49–60.
61. Bradshaw, H. D. Jr., Hollick, J. B., Parsons, T. J., Clarke, H. R. G. and Gordon, M. P., Systemically wound-responsive genes in poplar trees encode proteins similar to sweet potato sporamins and legume Kunitz trypsin inhibitors. *Plant Mol. Biol.*, 1989, **14,** 51–59.
62. Bradshaw, H. D. Jr., Parsons, T. J. and Gordon, M. P., Wound-responsive gene expression in poplars. *For. Ecol. Manage.*, 1991, **43,** 211–224.
63. Hood, C. E., Life history and control of the imported willow leaf beetle. *U.S. Dept. Agric. Circ.*, 1940, **572,** 1–9.
64. Wade, M. J. and Breden, F., Life history of natural populations of the imported willow leaf beetle, *Plagiodera versicolora* (Coleoptera : Chrysomelidae). *Ann. Entomol. Soc. Am.*, 1986, **79,** 73–79.
65. Hilder, V. A., Gatehouse, A. M. R., Sheerman, S. E., Barker, R. F. and Boulter, D., A novel mechanism of insect resistance engineered into tobacco. *Nature*, 1987, **330,** 160–163.
66. Johnson, R., Narvaez, J., An, G. and 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*, 1990, **86,** 9871–9875.
67. McManus, M. T., White, D. W. R. and McGregor, P. G., Accumulation of a chymotrypsin inhibitor in transgenic tobacco can affect the growth of insect pests. *Transgenic Res.*, 1994, **3,** 50–58.
68. Wolfson, J. L. and Murdock, L. L., Diversity in digestive proteinase activity among insects. *J. Chem. Ecol.*, 1990, **16,** 1089–1102.
69. Brattsten, L. B., Bioengineering of crop plants and resistant biotype evolution in insects: Counteracting coevolution. *Arch. Insect Biochem. Physiol.*, 1991, **17,** 253–267.