# Transgenic *Populus* hybrid expresses a wound-inducible potato proteinase inhibitor II – CAT gene fusion[1]

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A hybrid poplar clone has been transformed with a previously constructed wound-inducible potato proteinase inhibitor (*pin2*) – chloramphenicol acetyltransferase (CAT) chimeric gene linked to a nopaline synthase (*nos*) – neomycin phosphotransferase II (NPT II) selectable marker gene. The *Populus alba* × *Populus grandidentata* Hansen clone was transformed by means of leaf cocultivation with *Agrobacterium tumefaciens* strain A281 containing a binary vector. Shoots were regenerated and rooted on selective medium containing kanamycin sulfate. Subsequently, plants were established in soil for greenhouse and field growth. NPT II activity from the *nos* – NPT II selectable marker gene was observed in leaf extracts, thereby confirming expression of the transferred selectable marker gene in poplar. Southern hybridization confirmed the incorporation of a single copy of the *pin2*–CAT construction into the genome of the transformed hybrid poplars. Northern analysis of transgenic poplar leaves demonstrated that the potato *pin2*–CAT gene construction also was inducible in this woody dicotyledon. Thus, the wound-inducible promoter from an herbaceous dicot functions in a distinct family of woody dicots.

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Un clone de peuplier hybride a été transformé par une construction génétique constituée d'une fusion chimérique du gène inductible par la blessure codant pour l'inhibiteur de protéinase chez la pomme de terre (*pin2*) et du gène CAT (chloramphénicol acétyltransférase), liée au gène *nos* – NPT II (nopaline synthase – néomycine phosphotransférase II) marqueur de sélection. Le *Populus alba* × *Populus grandidentata* clone Hansen a été transformé au moyen de la co-culture de feuilles avec la souche A281 d'*Agrobacterium tumefaciens* contenant un vecteur binaire. Des tiges furent régénérées et enracinées sur milieu sélectif contenant du sulfate de kanamycine. Par la suite, les plants furent transférés en sol en vue de la croissance en serre et au champ. L'activité NPT II du gène *nos* – NPT II marqueur de sélection était observée dans les extraits de feuilles, confirmant donc l'expression du marqueur de sélection introduit chez le peuplier. L'hybridation Southern a confirmé l'incorporation d'une seule copie de la construction *pin2*–CAT au sein du génome des peupliers hybrides transformés. L'analyse Northern des feuilles de peupliers transgéniques a démontré que la construction génétique *pin2*–CAT était aussi inductible chez cette espèce dicotylédone ligneuse. Il en découle que le promoteur inductible par la blessure d'une espèce dicotylédone herbacée peut fonctionner chez une famille distincte de dicotylédones ligneuses.

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## Introduction

Plants respond to wounding by activating a variety of gene systems involved in defensive pathways. These gene systems are activated to foster the plants' return to a normal physiological state. Such gene systems close the wound and produce chemicals that inhibit the growth of potentially harmful herbivores (Ryan 1978) and invading microorganisms (Dixon 1986).

One of the best characterized plant defense gene systems is the proteinase inhibitor system of solanaceous plants (potato and tomato; Green and Ryan 1972; Ryan 1981, 1990). These plants contain genes that express in their foliage small, proteinaceous inhibitors of animal digestive enzymes (Ryan 1966). Accumulation of these inhibitors in foliage is induced by insect attack or other methods of mechanical wounding (Green and Ryan 1972, 1973). This accumulation occurs in about a day. Further, these plants respond systemically to local wounding (Ryan 1974).

The systemic response was initially characterized by wounding experiments with young tomato plants (Green and Ryan 1973). These experiments indicated that a transmissible factor, termed proteinase inhibitor inducing factor (PIIF), was formed or released in the wounded leaves and was transported into the unwounded leaves, where proteinase inhibitors were induced. It was further determined that the transmission half-life of the inducing factor was less than 4 h and that the signal probably was transported through the phloem (Zuroske et al. 1980). A phylogenetic survey for PIIF activity was conducted that covered 20 families of plants and fungi (McFarland and Ryan 1974). The universality of this defense-response inducer was indicated when PIIF-like activity was found in most plant species tested.

The complementary DNAs (cDNAs) (Graham et al. 1985*a*, 1985*b*; Sanchez-Serrano et al. 1986), as well as the genes for these proteinase inhibitors (Lee et al. 1986; Fox 1986; Cleveland et al. 1987; Thornburg et al. 1987*a*; Keil et al. 1986), have been isolated from both potato and tomato. The wound inducibility of the proteinase inhibitor II gene, *pin2*, in transgenic plants was demonstrated independently by two groups (Thornburg et al. 1987*b*; Sanchez-Serrano et al. 1987). In the earlier work, a chimeric gene was constructed consisting of the promoter of the wound-inducible *pin2* gene linked to the chloramphenicol acethyltransferase (CAT) gene. This chimeric gene was introduced into *Agrobacterium tumefaciens* (Smith & Townsend) Conn and then into *Nicotiana tabacum* L. cv. Xanthi (Thornburg et al. 1987*b*). The chimeric gene was not expressed in unwounded foliage but was expressed after wounding, thereby indicating that the wound-inducible activation of this potato gene was able to cross the species barrier into tobacco. We have herein extended this observation on the nature of this wound-signalling system in the plant kingdom to trees by transferring a potato *pin2*-CAT chimeric gene into the poplar genome, where it functions in an inducible manner.

## Materials and methods

### *Materials*

*Populus alba* L. × *Populus grandidentata* Michx. clone Hansen served as the parent material for our transformation studies. This clone is a natural hybrid that originated in southeast Iowa. Carbenicillin was from Roerig Pfizer, New York, New York, and Cefotaxime was from Hoechst-Roussel, Somerville, New Jersey. MS salts (Murashige and Skoog 1962) were purchased from Hazleton Biological Supply, Lexena, Kansas. All radioactive compounds and hybridization membranes were obtained from New England Nuclear, Boston, Massachusetts. Restiction enzymes were from Promega, Madison, Wisconsin.

The binary vector plasmid, pRT45, which contains the wound-inducible potato *pin2*-CAT and the nopaline synthase (*nos*) – neomycin phosphotransferase II (NPT II) constructions, was described previously (Thornburg et al. 1987*a*, 1987*b*). Other plasmids were used as probes in Southern and Northern analyses to verify the presence and expression of the *pin2*-CAT construction in the transgenic poplar plants. A plasmid containing the CAT gene, pGA425, was previously described (An 1987). Another plasmid, pRT55, containing some *pin2* coding sequences with its native 3′ flanking sequences, was subcloned as a 2.0-kilobase (kb) *Pst*I–*Sca*I fragment from the complete *pin2* gene into pUC13.

### *Establishment and maintenance of* Agrobacterium *cultures*

The binary vector plasmid, pRT45, was incorporated into *Agrobacterium* strain A281 containing the armed helper plasmid pTiBo542 (Hood et al. 1984) as previously described (Thornburg et al. 1987*a*, 1987*b*). All *Agrobacterium* cultures were maintained on MGL medium and manipulated according to established protocols (An 1987). The MGL medium was supplemented with 20 μg/mL kanamycin sulfate, 10 μg/mL tetracycline HCl, and 10 μg/mL acetylsyringone (Stachel et al. 1985).

### *Transformation of* Populus *by cocultivation*

The basic tissue-culture medium for poplar manipulations, MSB5, contained MS salts (Murashige and Skoog 1962), B5 vitamins (Gamborg et al. 1968), 3% sucrose, and 2.0 μg/mL glycine. Unless otherwise indicated, all plant media were solidified with 0.8% agar (Difco-Bacto). Hormones were added, and pH was adjusted to 5.6 before autoclaving. Antibiotics were filter sterilized separately before they were incorporated into the various permutations of the medium as described later.

*Populus alba* × *P. grandidentata* clones were established and maintained *in vitro* as previously described (Chun and Hall 1984; Chun et al. 1986). Rooted plantlets cultured for 8 weeks on rooting medium (MSB5 plus 0.2 μg/mL indole-3-butyric acid) served as the leaf source material for cocultivation. Leaves (1–2 cm long) were excised and placed in Petri dishes containing 30 mL cocultivation medium (MSB5 liquid medium plus 1.0 μg/mL 6-benzylaminopurine and 0.1 μg/mL α-naphthaleneacetic acid). To each 30 mL of cocultivation medium, 0.3 mL of *A. tumefaciens* (strain A281 containing the binary vector, pRT45) grown in MGL to an $OD_{550}$ of 0.5 was incorporated.

Leaf segments were cocultivated with *Agrobacterium* for 2 days in the dark at 22°C. After cocultivation, leaf segments were rinsed five times in sterile cocultivation medium, with the final rinse containing 500 μg/mL carbenicillin and 250 μg/mL cefotaxime. After rinsing, leaf segments were placed with the abaxial side in contact with nonselective shoot regeneration medium (MSB5 solid medium containing 500 μg/mL carbenicillin and 250 μg/mL cefotaxime and also containing the same hormones as the cocultivation medium). After 10 days of nonselective culture, leaf segments were again rinsed and then replated on selective shoot regeneration medium containing kanamycin (40 μg/mL). Untransformed control individuals of Hansen were regenerated as described earlier by using noncocultivated leaf explants. In all regeneration steps, untransformed control material was handled similarly to the cocultivated material, except that kanamycin was excluded from the culture medium.

All plant cultures, except those undergoing cocultivation, were maintained at 23 ± 2°C under cool-white, fluorescent lights emitting ca. 50 $\mu E \cdot m^{-2} \cdot s^{-1}$ with a 16-h light cycle. After 10–24 days on the selective medium, shoots were produced. These regenerated shoots were placed onto MSB5 selective rooting medium containing 0.2 μg/mL indole-3-butyric acid and 20 μg/mL kanamycin sulfate for secondary selection. Rooted plantlets were propagated,

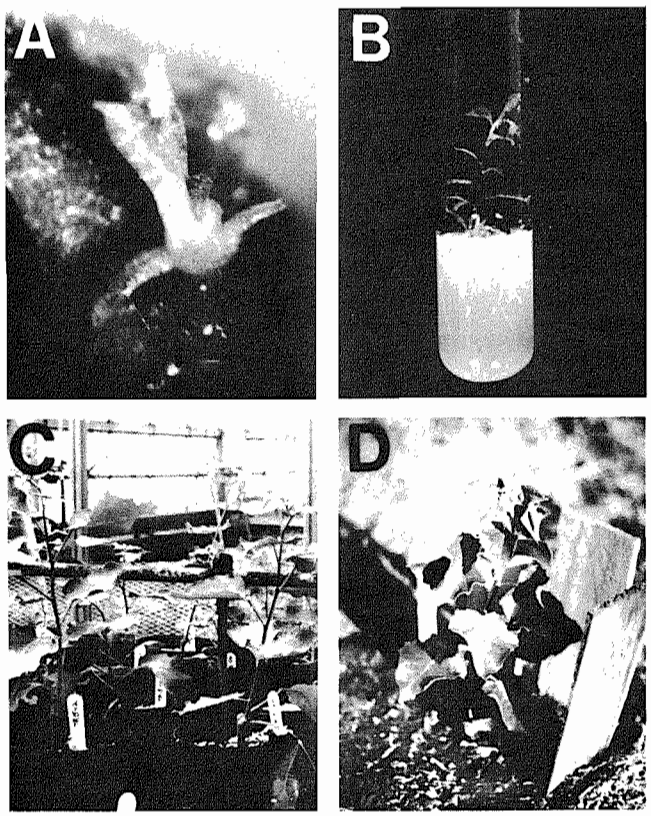



FIG. 1. (A) Regeneration of poplar hybrids containing pRT45. After cocultivation of poplar leaf pieces with *A. tumefaciens* strain A281 harboring pRT45, transformants were selected as described in Materials and methods. (B) Rooting of plantlets obtained from cocultivation. Selected plantlets were propagated, rooted, and adapted to soil *in vitro* before transfer into pots. (C) The selected trees were habituated to lower humidity levels in greenhouse environments for use in expression studies. (D) Multiple propagants of Tr15 were outplanted to a field site near Ames, Iowa, in accordance with USDA - Animal and Plant Health Inspection Service permit number 89-109-03.

rooted, and established in artificial soil mix *in vitro*, then gradually adapted for growth in the greenhouse.

*Plant analysis*

*Neomycin phosphotransferase II assay*

The oldest leaves of the transgenic plants were excised and homogenized in 1 mL ice-cold extraction buffer as described previously (Thornburg et al. 1987*b*). Nondenaturing polyacrylamide-gel electrophoresis and assays of NPT II activity were performed as described by Reiss et al. (1984) by using 20 μg extracted plant protein (Bradford 1976).

*Southern analysis*

Total poplar DNA was isolated from fresh leaf tissue by following the protocol of Rogers and Bendich (1988). This procedure was employed to reduce contaminating polysaccharides that have been reported to interfere with restriction endonuclease digestion and gel electrophoresis of poplar DNA (Fillatti et al. 1987). This method of DNA extraction provided poplar DNA of sufficient purity to allow Southern analysis. Restriction enzyme analysis was performed under conditions suggested by the supplier. After electrophoresis and blotting to GeneScreen, the nylon membranes were hybridized at 65°C with random primed $^{32}$P-labelled probes according to standard procedures (Sambrook et al. 1989).

*Wound induction*

Excised leaves were gently agitated at room temperature in liquid basal MSB5 medium containing 3% sucrose. The leaves were wounded with hemostats at times 0, 8, and 16 h as previously described (Graham et al. 1986; Johnson and Ryan 1990), and *pin2*-CAT gene expression was analyzed at 24 h.

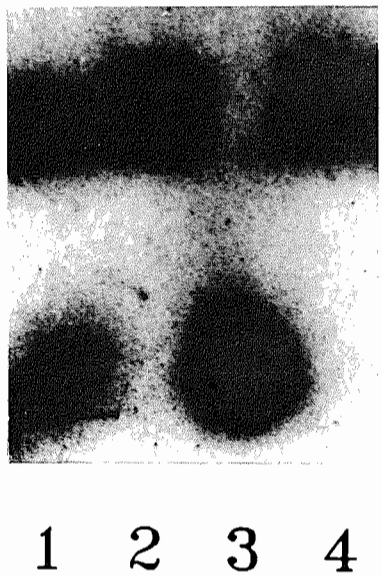



FIG. 2. NPT II assays. Leaves of poplar plants at the stage of Fig. 1C were processed for NPT II assay according to the methods outlined in the text. Lane 1 is the transgenic poplar clone, Tr15, transformed with pRT45; lane 2 is poplar Tr19B that has passed the initial two screenings on kanamycin; lane 3 contains 1 μg of protein extracted from *A. tumefaciens* containing the binary vector, pRT45; and lane 4 represents an untransformed Hansen clone. Spots toward the bottom of lanes 1 and 3 are representative of NPT II activity.

*Northern analysis*

RNA was extracted from wounded and unwounded leaves using the procedures of Dean et al. (1985). Northern blotting was performed by following previously described procedures (Ausubel et al. 1987). RNAs were separated on formaldehyde-agarose gels and transferred to nitrocellulose membranes, and the blot was probed at 42°C with 50% formamide with a random primed *Bgl*II-*Bam*HI fragment of pGA425, which contains the full length coding sequence of the CAT gene. Autoradiography was performed at −70°C using an intensifying screen.

## Results

*Production of transgenic poplar trees*

Cocultivation of *A. tumefaciens* A281 harboring the wound-inducible potato *pin2*-CAT gene fusion yielded many regenerable shoots on our selection medium. In a typical experiment, cocultivated leaf explants of Hansen produced an average of 2.3 shoots per explant on selective regeneration medium. Because these experiments utilized a wild type *A. tumefaciens* strain, A281, these same cocultivation experiments also produced 2.7 calli per explant. Callus tissue did not regenerate and is believed to originate from dividing cells transformed with both wild-type transfer DNA (T-DNA) from pTiBo542 and marker genes from the binary vector. Figure 1A shows a typical poplar leaf explant undergoing regeneration on selective medium. Regenerated shoots were viable and typically remained bright green, and the parental explant browned, darkened, and eventually died. After approximately 24 days on selective medium, shoots typically were 1–1.5 cm high. At this point, the regenerated

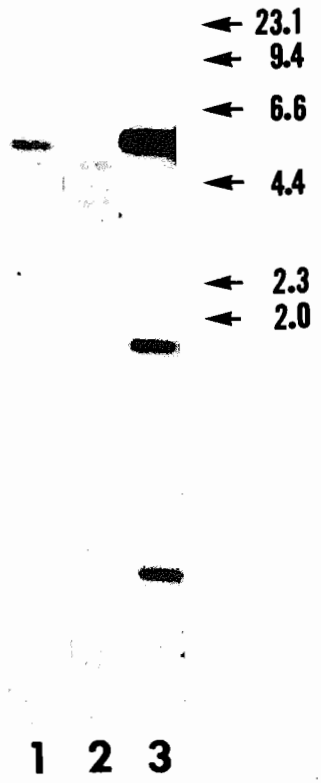



FIG. 3. Southern blot analysis of transformed poplar plants. Total DNA extracted from the foliage of the transgenic Hansen clone, Tr15, (lane 1) or untransformed Hansen clone (lane 2) was restricted with *Hin*dIII, and 10 μg of each digest was electrophoresed, blotted, and probed with a segment of pRT55 bearing the coding and 3′ flanking sequences of *pin2*. Lane 3 is a control lane containing pRT45 digested to completion with *Hin*dIII and partly digested with *Eco*RI to generate a molecular size ladder containing *pin*2 sequences hybridizing at 5.3, 2.7, and 1.4 kb. The indicated markers represent a *Hin*dIII cut bacteriophage λ ladder. Molecular sizes are given in kilobases.

shoots were transferred to a rooting medium containing a lowered kanamycin level for secondary selection.

Even though no adventitious shoots or calli developed from the noncocultivated, control leaf explants on selective medium, we employed a selective rooting medium as a secondary selection step to help screen out possible escapes from primary selection. We empirically determined that 20 μg/mL kanamycin completely inhibited root development in untransformed Hansen shoots; therefore, we incorporated this concentration of kanamycin into the rooting medium. Using this selective rooting medium, we were able to reduce the numbers of selected plantlets by 85%.

After 10–30 days for root initiation and growth, rooted plantlets (Fig. 1B) were propagated *in vitro* and transferred to capped culture vessels containing moist, artificial soil mix (peat, perlite, and vermiculite in equal parts). Plantlets gradually were adapted to lower humidity conditions over 2–3 weeks, then repotted into 10-cm pots and adapted for growth-chamber and greenhouse growth (Fig. 1C). After 2 months growth in pots, the transformed poplar plants reached a height of 15–20 cm and had produced at least

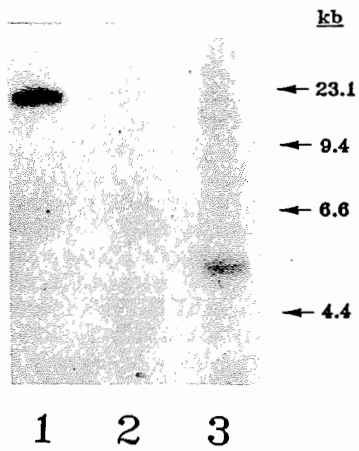



FIG. 4. Integration of CAT gene sequences into poplar genome. DNA was isolated from the parent plasmid, pRT45 (lane 1, containing 1 ng), an untransformed Hansen poplar clone (lane 2, containing 10 μg), or the transformed poplar clone, Tr15 (lane 3, containing 10 μg). Each DNA was digested to completion with *Sma*I, then electrophoresed on a 0.8% agarose gel. The electrophoresed DNAs were blotted to a nylon membrane and probed with the coding region for the CAT gene.

10 expanded leaves (ca. 4 × 6 cm). Plant lines were subsequently cloned by nodal propagation (Faltonson et al. 1983). Multiple propagants of Tr15 were outplanted to a field site near Ames, Iowa, on 28 July 1989 in accordance with USDA – Animal and Plant Health Inspection Service Biotechnology Permit Unit guidelines (permit 89-109-03) (Fig. 1D).

*Plant analysis*

After plants were growing in pots, we began our analysis of the transgenic plants. Simplified methods for detecting NPT II activity in crude protein extracts (McDonnell et al. 1987) did not yield consistent results with our poplar tissues. Therefore, more specific methods involving nondenaturing polyacrylamide-gel electrophoresis were employed (Reiss et al. 1984). Figure 2 illustrates one such NPT II analysis that demonstrates a positive poplar transformant; compare the transgenic poplar clone (Tr15; lane 1) with protein extracted from the pRT45 containing *A. tumefaciens* (lane 3). The untransformed Hansen poplar clone (lane 4) showed no NPT II activity. Protein preparations from a substantial proportion of poplar plantlets that had passed both kanamycin screens did not show evidence of NPT II activity (lane 2).

Plants that consistently demonstrated NPT II activity were used for additional assays of gene insertion and expression. To demonstrate the presence of the transferred DNA in the regenerated poplar trees, we isolated total DNA for Southern blot analysis (Southern 1975). The results of a typical Southern blot analysis probed with sequences containing the *pin2* terminator region are shown (Fig. 3). The control lane (lane 3) is pRT45 digested to completion with *Hin*dIII and partially digested with *Eco*RI to generate a molecular size ladder containing *pin2* sequences hybridizing at 5.3, 2.6, and 1.3 kb. A *Hin*dIII digest of the transgenic poplar (Tr15) DNA (lane 1) generated a hybridizing fragment that comigrated with the 5.3-kb fragment in the control lane. This band was not present in the DNA of untransformed poplar (lane 2); however, we frequently

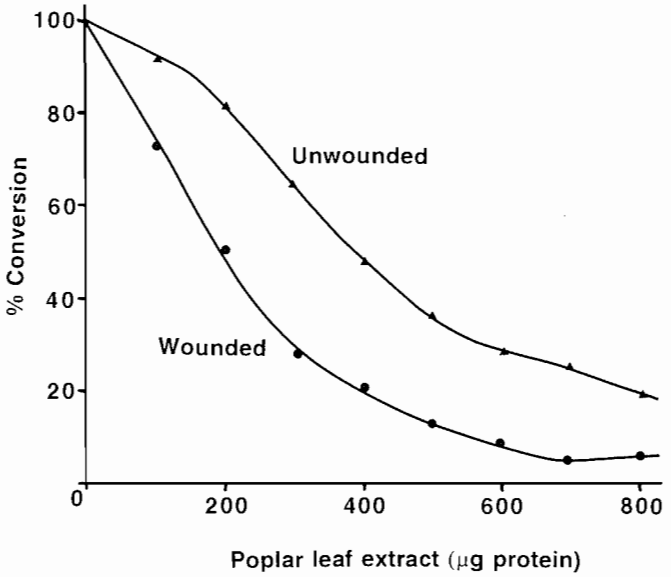



FIG. 5. Ten units of *E. coli* CAT protein were reacted for 20 min at 37°C in the presence of leaf extracts of either unwounded or wounded untransformed Hansen leaves. Following the reaction, the chloramphenicol acetates were extracted and run on TLC plates as described in Materials and methods. The TLC plates were cut and counted to quantitate the amount of unreacted [$^{14}$C]-chloramphenicol and acetylated [$^{14}$C]chloramphenicol. The data are presented as the percentage of conversion of chloramphenicol into the acetylated forms.

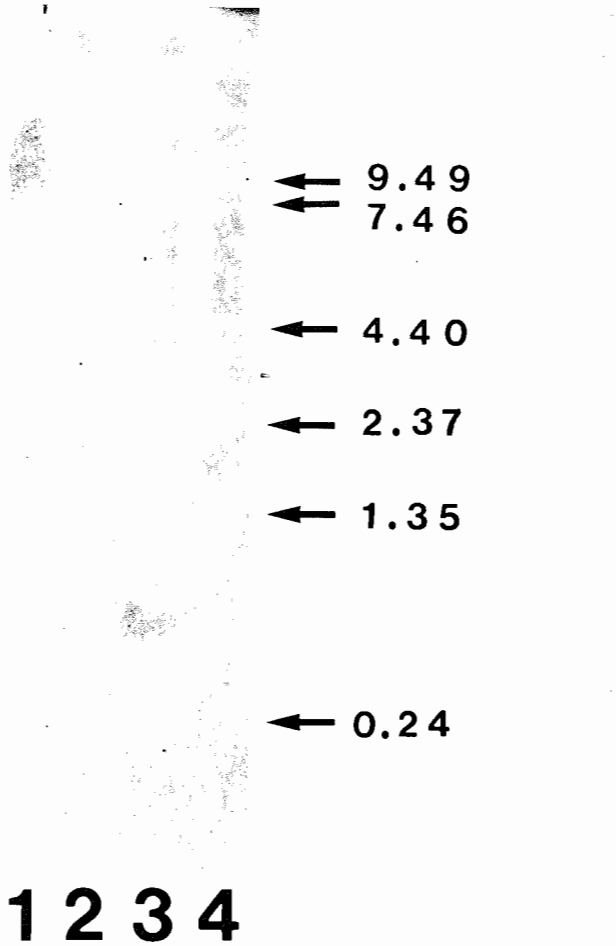



FIG. 6. Northern blot analysis of untransformed and transformed poplar plants. Leaves were obtained from either untransformed regenerated Hansen clones (lanes 1 and 2) or transformed Tr15 Hansen clones (lanes 3 and 4) for Northern blot analyses. Some of the leaves (lanes 2 and 3) were wounded and induced *in vitro* as described in Materials and methods. Total RNA was extracted from these tissues and 20 μg applied to each lane. After formaldehyde – agarose gel electrophoresis and transfer to nitrocellulose, the blot was probed with the *Bam*HI–*Bgl*II insert of pGA425, which contains the full length coding sequence of the CAT gene.

observed weakly hybridizing bands in the 4- to 5-kb range in the DNA both untransformed and transgenic poplar trees (see lanes 1 and 2). These bands may represent nonspecific hybridization to abundant DNAs such as those present in digests of chloroplast, mitochondrial, or highly repetitive nuclear DNAs.

To demonstrate integration of the T-DNA into the genome of the transgenic poplars, we performed additional Southern blot analyses. DNA from the binary vector, pRT45, and from the leaves of untransformed and transgenic poplars was digested with *Sma*I. This enzyme cuts at a single location inside the T-DNA of the binary vector, pRT45. As shown in Fig. 4, *Sma*I linearizes the plasmid, pRT45, giving a single band the size of the full length binary vector. Untransformed poplar DNA shows no hybridization with the CAT probe; however, DNA from the transgenic Hansen clone, Tr15, hybridizes with only a single band. This 5.5-kb fragment corresponds to the left border region of the T-DNA from pRT45 and extends approximately 0.2 kb into the poplar chromosomal DNA. The absence of additional bands indicates that only a single copy of the T-DNA was inserted into the genome of Tr15.

In as much as transformant Tr15 possessed the kanamycin selectable marker (by NPT II assay) and the *pin2* sequences (by Southern analysis), we initiated a series of tests to examine the expression of the *pin2*-CAT gene in this transgenic poplar clone. Using standard procedures of CAT activity analysis (Gorman et al. 1982), we repeatedly were unable to demonstrate the presence of a wound-inducible functional CAT enzyme in these transgenic plants. Controlled experiments using crude extracts from leaves of untransformed poplars indicated that components in poplar extracts interfered with the CAT assay (Fig. 5). The nature of these components has not been elucidated fully, but their effect increases upon wounding of the poplar leaves. When extracts of unwounded and wounded poplar leaves were included in CAT assays with standard amounts of purified *Escherichia coli* – CAT protein, the wounded-leaf extracts consistently inhibited the CAT protein activity more than did the extracts of unwounded poplar leaves. In addition, similar experiments with other untransformed poplar hybrids, *Populus deltoides* Bartr. ex Marsh. × *Populus nigra* L. and *P. deltoides* × *Populus maximowiczii* Henry, have demonstrated a similar inhibition of CAT activity (N.B. Klopfenstein, unpublished results). Various attempts to circumvent the inhibition of CAT activity by methods such as increasing the antioxidant capacity of the extraction buffer, heating the protein extracts to 65°C, increasing the acetylcoenzyme A concentration in the reaction mixture, or attempting to elicit CAT gene expression without wounding have thus far proved unsuccessful. Although we have yet to determine the exact cause of the interference, likely candidates could involve an active

acyl hydrolase or, perhaps, chalcone synthase, which would scavenge acetylcoenzyme A from the CAT assay reaction mixture. Alternative explanations could involve proteinases that might inactivate the CAT enzyme or various phenolic compounds, which are plentiful in *Populus* spp.

Because the CAT assay does not serve as a useful indicator of CAT gene expression in the transgenic poplar trees, we examined mRNA levels by Northern analysis. CAT specific mRNAs were monitored in leaves of Tr15 and a regenerated untransformed Hansen clone that were wounded and further induced *in vitro* as described in Materials and methods. There is an increase in mature mRNA coding for CAT protein in the transgenic poplar clone, Tr15, following wounding (Fig. 6, compare lanes 3, wounded, and 4, unwounded). Based upon the indicated molecular size standards, this mRNA is approximately 800 nucleotides in size. This is the expected size of mRNA arising from transcription of the *pin2*–CAT gene fusion. In regenerated, untransformed Hansen clones, neither unwounded (lane 1) nor wounded (lane 2) leaf tissues express a hybridizing band. The Northern analysis was repeated four times with comparable results.

## Discussion

We initially began this study to determine whether a woody dicotyledon could express specific, regulated genes derived from a herbaceous dicotyledon. The inhibitor II gene system that we have been using is an ideal candidate for these studies because it is derived from a potato gene that is regulated in the foliage by wounding (Thornburg et al. 1987*a*, 1987*b*). This construction contains both the 5′ and the 3′ flanking regions of the *pin2* gene fused to the coding region of the *E. coli* CAT gene.

Poplar is advantageous for studies on the regulation of expression of introduced genes because species of *Populus* demonstrate developmental plasticity in their response to tissue-culture manipulations, such as *in vitro* micropropagation of axillary shoots and regeneration from protoplasts, calli, leaf, stem, or root segments (Chun et al. 1988*a*; Son and Hall 1990). In addition, *Populus* spp. are amenable to transformation by *Agrobacterium*-based systems (Fillatti et al. 1987; Chun et al. 1988*a*, 1988*b*; Parsons et al. 1986; Pythoud et al. 1987). Another advantage is that *Populus* spp. possess relatively small genomes ($c$ = 0.7 pg), which facilitates screening at the molecular level (Dhillon et al. 1984; Parsons et al. 1989).

We have transformed hybrid poplar trees with a wound-inducible chimeric gene derived from a potato *pin2* gene and demonstrated that this gene is functional in the transgenic trees. The culture methods that we used to produce the transgenic poplar trees are somewhat simpler than those previously published by Fillatti et al. (1987). These workers utilized a tobacco cell feeder layer to provide the necessary conditions for regeneration of poplar shoots, but comparable regeneration rates were obtained with our simplified culture methods. Using our described cocultivation conditions with the armed *Agrobacterium* binary vector system, we have observed that a 1- to 2-day cocultivation period is optimal for shoot regeneration and that longer cocultivation increases the proportion of callus.

To evaluate whether wild-type T-DNA was transferred to the transgenic Tr15 poplar clone along with binary vector T-DNA, we utilized the procedures of Parsons et al. (1986). These tests indicated that agropine production was not present in the transgenic clone, Tr15. This provides evidence that wild-type T-DNA was not integrated into the genome of Tr15.

We were unable to detect CAT enzyme activity in transgenic poplar by using standard methods. Seemingly, an unknown substance present in poplar leaf extracts interferes with the CAT assay. Although this inhibiting substance has not been characterized, it is itself wound activated. The inability to detect CAT activity in poplar leaves by the simple methods of assaying enzyme activity reduces the utility of CAT as a reporter gene in *Populus*. Current efforts are being directed toward assessing the nature of the inhibitory activity, as well as examining *pin2*–CAT gene expression in transgenic poplar by western blotting.

Although we could not detect CAT activity, we were able to confirm integration of one copy of the CAT gene and detect wound-inducible CAT mRNA in the transgenic poplar. Thus, we were able to demonstrate that a wound-inducible gene derived from potato, a herbaceous dicotyledon, is able to function in poplar, a woody dicotyledon. Wounding is a common problem for which all plants must have the capacity for effective response. Because most bacterial and fungal pathogens are opportunistic and enter plants after wounds (Layne 1967), the inability of a plant to respond to wounding would likely be very deleterious. Some of the mechanisms that plants use to respond to wounding seem to be conserved across most of the plant kingdom (McFarland and Ryan 1974). Further, wound-inducible cDNAs were recently isolated from the wounded foliage of poplar trees (Parsons et al. 1989). The details of the wound-induction mechanisms are not sufficiently understood, however, to permit predictions of gene function between highly diverged families as the Solanaceae and Salicaceae. The data presented herein indicate that at least some of the mechanisms of gene activation for wound-inducible genes are broadly conserved. It would be of interest to examine whether poplar trees possess other biochemical features that are associated with proteinase inhibitor induction in the Solanaceae, such as polygalacturonase induction (Bishop et al. 1981), PIIF activity (Bishop et al. 1984), or auxin repression (Kernan and Thornburg 1989).

Tree-growth and wood-fiber losses caused by insects and diseases are limiting factors in the success of poplar crop systems (Ostry et al. 1989). Genetic engineering potentially can augment woody crop resistance to pest problems by introducing novel pest-resistance genes into the woody plant genome (McNabb et al. 1990). It is necessary, therefore, to consider carefully the predicted stability of introduced resistance genes because of the multiyear nature of woody crop systems and their high cost of establishment. In addition, the regulation of pest-resistance gene expression also must be considered when attempting to genetically engineer pest resistance in these crops.

Improved resistance stability and reduced physiological cost to the plant can be attained through understanding the regulation of introduced pest resistance genes to a point at which they are expressed only when plants are subjected to specific thresholds of pest damage (Gould 1988; Raffa 1989; Thornburg 1990). The activity of the wound-inducible *pin2* promoter in poplar trees demonstrates the potential to regulate efficiently chimeric resistance genes in woody crops.

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