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Transformation of Populus Hybrids to Study and Improve Pest Resistance[1])

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Summary

Plant defense mechanisms that have activity against a specified pest contribute to resistance of trees to that pest, provided that the defense mechanism is expressed at adequate levels in the appropriate tissues. Additionally, the stability and (or) physiological efficiency of the resistance may be increased by expressing the defense mechanism only when the tree is threatened by pest attack or challenge. In studies aimed at understanding and improving pest resistance of trees, 2 *Populus* hybrids, *Populus alba* L. X *P. grandidentata* MICHX. and *P.* X *euramericana* (DODE) GUINIER, were transformed with chimeric plant defense gene constructs based on the potato proteinase inhibitor II (*pin2*) gene. An *Agrobacterium* binary vector system was used to transform these hybrids with one of the following chimeric genes: 1) a wound-inducible *pin2* promoter linked to a chloramphenicol acetyltransferase (*CAT*) reporter gene; 2) a bacterial nopaline synthase (*nos*) promoter linked to a *PIN2* structural gene; or 3) a cauliflower mosaic virus *35s* promoter linked to a *PIN2* structural gene. All of the transgenic poplars also were transformed with a selectable marker gene consisting of a *nos* promoter linked to a neomycin phosphotransferase II (*NPT II*) structural gene which confers kanamycin resistance.

Tissue-specific expression of the *nos-NPT II* gene construct is being evaluated with enzyme-linked immunosorbent assays (ELISAs). Transgenic poplar lines from separate transformation events demonstrate variable

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levels of *nos-NPT II* expression. Expression of *nos-NPT II* was detected in leaves, petioles, stems, and roots of one transgenic poplar (Tr15). Thus, the *nos* promoter has the potential to regulate chimeric defense genes in poplar when constitutive, whole-plant expression is warranted.

Assays of the *CAT* reporter gene were hampered by a component of wounded poplar leaf extracts that inhibits CAT enzyme activity. Nevertheless, inducible expression of the *pin2-CAT* gene construct was demonstrated by northern hybridization, indicating that *pin2* has the potential to promote expression of introduced defense genes in response to pest attack or challenge. An established field test of transgenic poplars containing *pin2-CAT* has completed its third season, and is being evaluated for transgene expression and effects of gene insertion on growth.

Efforts to confirm transgene expression in poplars transformed with *nos-PIN2* or *35s-PIN2* currently are underway. Transgenic poplars expressing *PIN2* will be evaluated for resistance to insects (imported willow leaf beetle, *Plagiodera versicolora* LAICHARTING, and cottonwood leaf beetle, *Chrysomela scripta* F.) and fungal pathogens (*Septoria musiva* PECK. and *Melampsora medusae* THUM.).

Key words: Populus, Agrobacterium, proteinase inhibitor, pest resistance, genetic engineering, neomycin phosphotransferase.

Introduction

Molecular biological studies of defense gene systems and their regulation in woody plants offer a unique opportunity to augment programs aimed at improving tree growth and survival (ERNST and KLOPFENSTEIN, 1990). Damage caused by diseases and insects frequently is the limiting factor in tree growth and survival, especially when stress acts as a predisposing or interacting agent. Improvement of pest resistance in trees by conventional breeding and selection is a long-term process that frequently requires decades and has difficulty keeping pace with constantly changing pest problems. Intensive management of tree pest problems with pesticides typically is not economically feasible or environmentally sound. Molecular studies aimed at understanding the genetic mechanisms of host resistance can facilitate the development of pest-resistant trees in 2 ways: 1) the characterization of plant defense genes and regulatory systems can generate information that allows development of molecular probes to assist in the rapid selection of resistance traits, and 2) woody plant defense systems potentially can be bolstered by genetic transformation with identified pest resistance genes and regulatory sequences. These molecular techniques also allow the comparison or transfer of gene sequences across species boundaries.

The genus *Populus* is well-suited to serve as a model system for molecular genetic studies of woody plants. Species and hybrids of *Populus* are grown and valued worldwide for wood products, fiber, energy, and environmental protection. *Populus* spp. demonstrate developmental plasticity in their response to tissue culture manipulations, such as micropropagation of axillary shoots, and regeneration from protoplasts, calli, leaf, stem, or root segments (CHUN et al., 1986, 1988a, 1988b; SON and HALL, 1990). In addition, *Populus* spp. are amenable to transformation by *Agrobacterium*-based systems (CHUN et al., 1988a, 1988b; FILLATTI et al., 1987; KLOPFENSTEIN et al., 1991; PARSONS et al., 1986; PYTHOUD et al., 1987). The relatively small genome (c = 0.7 pg) of *Populus* spp. also greatly facilitates screening at the molecular level (DHILLON et al., 1984; PARSONS et al., 1989).

The effectiveness of a plant defense gene depends on its ability to be expressed in a controlled spatial and temporal manner with activity against the appropriate pest. Regulatory processes of defense genes must be understood before pest resistance of woody plants can be improved by identifying, characterizing, or transferring plant defense-related gene sequences. The initial conditions that foster defense gene expression, as well as conditions that suppress such expression, must be determined before these processes are understood. The relationship of tissue type and developmental stage to defense gene expression must also be determined. In addition, specific information is needed on the action of defense gene products against pests.

Genes encoding proteinase inhibitors in solanaceous plants are being studied because of their putative role in pest resistance. The expression of these inhibitor genes in foliage was found to be induced by mechanical wounding. One such gene from potato, proteinase inhibitor II (*pin2*), has been well characterized (RYAN and AN, 1988; THORNBURG et al., 1987a). When the flanking 5' regulatory sequences of the *pin2* gene were fused to a chloramphenicol acetyltransferase (*CAT*) reporter gene and transferred to tobacco or hybrid poplar, the chimeric gene was not expressed in unwounded foliage but was expressed in foliage following mechanical wounding. Therefore, a potato *pin2*-regulated gene was expressed in a wound-inducible manner in another herbaceous genus, *Nicotiana*, and a woody genus, *Populus* (KLOPFENSTEIN et al., 1991; THORNBURG et al., 1987b, 1990).

Work with potato and tobacco also has demonstrated that *pin2* is induced systemically by wounding (THORNBURG et al., 1987b). Further work has indicated that a transmissible factor, termed proteinase inhibitor inducing factor (PIIF), is formed in wounded leaves of solanaceous plants and transported to nonwounded leaves, thus allowing systemic induction of proteinase inhibitor genes (GREEN and RYAN, 1972; RYAN, 1974). Recently, a polypeptide-signaling molecule that initiates signal transduction to regulate plant defense genes has been characterized from tomato (PEARCE et al., 1991). Whether a woody plant such as hybrid poplar possesses the appropriate inducing factors to allow the systemic induction of the *pin2* promoter has not been determined.

Additional work by others with potato and tobacco has demonstrated that *pin2* also is activated by fungal cell wall components and by plant cell wall components that are liberated by cell wall degrading enzymes (RYAN, 1988). A similar wound-inducible gene (*wun1*) isolated from potato has been shown to be induced following infection by the phytopathogen *Phytophthora infestans* (LOGEMANN et al., 1989). Thus, a potential role of the *pin2* promoter in regulating disease-resistance genes is demonstrated; however, a role of *pin2* in disease resistance is yet to be established for woody plants.

When considering putative plant defense genes for transfer to woody plants, concerns include target pests and predicted stability of the imparted resistance trait. The *pin2* gene encodes an inhibitor of the protein degrading enzymes, trypsin and chymotrypsin. These digestive enzymes are the major endopeptidases of phylogenetically diverse animals (including herbivorous insects) and micro-

organisms (including plant pathogens) (Broadway and Duffey, 1986; Mosolav et al., 1976). Inhibitors of these proteinases are thought to provide foliage with antinutrient properties that prevent organisms from obtaining adequate nourishment from the plant tissues (Broadway et al., 1986). In theory, this process should retard development and reproduction of insects and pathogens without causing direct mortality. A strong selection pressure that can cause a rapid buildup of insect and pathogen resistance to a defense mechanism is thereby avoided (Gould, 1988; Raffa, 1989; Thornburg, 1990).

We are using genetic transformation of *Populus* spp. in our efforts to understand and improve woody plant defense systems. Studies of promoter activity will determine environmental, spatial, and temporal regulation of introduced gene constructs. These transgene expression studies will provide a basis for developing and selecting efficient regulation systems for defense genes in poplar. In addition, poplars transformed with chimeric genes containing the *PIN2* structural region will be used to characterize the role of proteinase inhibitors in pest resistance. Parallel studies of pest biology will evaluate the relationship between a pest's ability to cause damage to poplars and its production of proteinases.

Research Progress

Transformation of Populus

An *Agrobacterium* binary vector system was used to transform leaf discs from Hansen and Crandon clones of *Populus alba* X *P. grandidentata* hybrids, and the Ogy clone of a *P. X euramericana* hybrid. *Agrobacterium tumefaciens* strains A281 containing the supervirulent pTiBo542 helper plasmid, or EHA101 containing a disarmed helper plasmid were used as vectoring agents for the *Populus* transformation. Within the transfer DNA, all binary vector plasmids contained a bacterial nopaline synthase (*nos*) promoter linked to the neomycin phosphotransferase II (NPT II) structural gene to provide a selectable marker (resistance to kanamycin). In addition, binary transfer DNA contained one of the following constructs based on the proteinase inhibitor II gene: 1) a wound-inducible *pin2* promoter linked to a *CAT* reporter gene; 2) a *nos* promoter linked to a *PIN2* structural gene; or 3) a cauliflower mosaic virus *35s* promoter linked to a *PIN2* structural gene.

Transgenic poplar shoots were regenerated from leaf discs on selective media containing 40 mg/L kanamycin. Putatively transformed shoots were subjected to a secondary screen for rooting ability on rooting media containing 20 mg/L kanamycin. Regenerated plantlets that had passed both kanamycin screens were established for greenhouse growth. Initial confirmation of transformation was achieved using an assay for NPT II enzyme activity following nondenaturing polyacrylamide-gel electrophoresis. Southern hybridization confirmed the presence of the transfer DNA in the poplar genome (Klopfenstein et al., 1991).

Expression of nos-NPT II in Populus

NPT II expression was initially determined by nondenaturing polyacrylamide-gel electrophoresis and assays of NPT II enzyme activity (Klopfenstein et al., 1991; Reiss et al., 1984). Such assays were effective, but were tedious, frequently difficult to reproduce, and involved relatively high levels of radioisotopes.

Subsequent efforts to monitor *NPT II* expression have used enzyme-linked immunosorbent assays (ELISA's) to detect tissue specific expression in three different lines of greenhouse-grown, transgenic Hansen. Leaves of three different physiological ages based on the Leaf Plastochron Index (Larson and Isebrands, 1971) (LPI's of -1 to 3, 9 to 13, and 19 to 23), petioles (at LPI 9 to 13), stem tips (at LPI -1 to 3), outer stems (outside of phloem at LPI 9 to 13), inner stems (inside of phloem at LPI 9 to 13), and roots were assayed for *NPT II* expression. Expression of *nos-NPT II* was variable among tissues and among plant lines arising from separate transformation events; however, in transgenic Hansen poplar Tr15, *nos-NPT II* expression was detected in all tissues tested. Thus, the *nos* promoter is of potential use for expressing chimeric defense genes in poplar when a general expression is warranted.

Inducible Expression of pin2-CAT in Populus

The *pin2-CAT* gene fusion was transferred to hybrid *Populus* to produce a woody plant system that contains an introduced gene under the control of an inducible promoter (Klopfenstein et al., 1991). Inducible expression of *pin2-CAT* was confirmed in transgenic poplar, Tr15, by northern hybridization; however, traditional assays of *CAT* expression were ineffective because components in the wounded poplar leaf extract inhibited CAT enzyme activity.

The inducible expression of the *pin2-CAT* gene in poplar demonstrates that the *pin2* promoter is able to function in a woody plant family, Salicaceae, that is quite distinct from the herbaceous family of its origin, Solanaceae. The wound inducibility of *pin2* in poplar demonstrates the potential for efficiently regulating resistance genes in woody plants to augment existing defense systems. Future work will be directed toward characterizing pathogen elicitation of *pin2* promoter activity. In addition, systemic induction of the *pin2* promoter will be evaluated to allow comparison with native wound-inducible genes of *Populus* spp. (Bradshaw et al., 1989; Davis et al., 1991; Parsons et al., 1989).

Field Studies of Transgenic Populus

Multiple propagants of untransformed Hansen controls and transgenic Hansen Tr15, a transformant with the *nos-NPT II* and *pin2-CAT* constructs (Klopfenstein et al., 1991), were outplanted to a field site near Ames, Iowa on July 28, 1989 in accordance with USDA/APHIS Biotechnology Permit Unit guidelines (Permit number 89-109-03) to study environmental and developmental effects on gene regulation and extended (multi-year) growth of these transgenic trees (McNabb et al., 1991). Height and diameter growth have been monitored at the end of each growing season. There have been no consistent differences in height growth between the transgenic and control plants. At the end of the third growing season, height averaged 517 cm and 519 cm respectively. Diameter growth of the transgenics has been consistently less than the controls, but the difference is not statistically significant. In general, it does not appear that this genetic transformation has had much impact on early growth.

Expression of nos-PIN2 and 35s-PIN2 in Populus

To evaluate possible effects of *PIN2* on pest resistance of woody plants, hybrid poplars were transformed with gene constructs containing the *PIN2* structural gene under the control of two different promoter regions, cauliflower mosaic virus *35s* or *nos*. At present, several

putatively transformed plantlets of hybrid poplar have passed initial screens indicating that a linked selectable marker gene, *NPT II*, has been transferred and is functioning (KLOPFENSTEIN et al., 1989; MCNABB et al., 1990). Before pest resistance studies are validated, however, translation of the transferred *pin2* gene must be confirmed with western analysis or ELISA.

Pest Resistance Studies

Preliminary studies at Iowa State University have demonstrated that two poplar insect pests, cottonwood leaf beetle (*Chrysomela scripta* F.), and imported willow leaf beetle (*Plagiodera versicolora* LAICHARTING) both produce proteinases that are inhibited by the *PIN2* gene product (R. W. THORNBURG and K. K. ALLEN, unpublished data). Laboratory/growth chamber, single-leaf feeding techniques have been developed for these insects, allowing tests of resistance imparted by introduced *PIN2* genes in transgenic poplar. Feeding studies are being conducted with *P. versicolora* on transgenic and control HANSEN to evaluate effects on larval development time, pupal weight, and leaf area consumed. Preliminary evidence indicates differences among transgenic and control HANSEN; however, correlations with levels of *PIN2* gene expression are still being verified.

The Septoria leaf-spot and stem-canker pathogen (LULEY and MCNABB, 1989, 1991; LULEY et al., 1987; NIYO et al., 1986) has been tested to determine if this disease-causing organism produces proteinases and whether the proteinases are inhibited by purified proteinase inhibitor II protein (SILLICK et al., 1989). Proteinase production was demonstrated for 2 different pathogen isolates. In addition, highly purified proteinase inhibitor II protein inhibited proteinase activity of the Septoria pathogen under the assay conditions. Studies are underway to determine the effect of the *PIN2* gene system on pathogen infection and development.

Application of Results

Information generated by the study of the *pin2* promoter and structural gene in poplar can be applied toward the evaluation and design of defense gene constructs for transfer into woody species to study and improve pest resistance. The added understanding of *PIN2* gene function in poplar can provide a basis for the characterization of additional defense genes in woody plants, as well as elucidate potential regulatory mechanisms involved in controlling the expression of such defense genes. The identification and characterization of suitable plant defense gene systems will provide information that can be integrated into conventional breeding programs to produce more pest-resistant trees.

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Somatic Embryo Induction and Germination in Quercus suber L.[1])

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Abstract

Embryogenic callus was formed on the hypocotyl of immature zygotic embryos under the influence of 2,4-dichlorophenoxyacetic acid (2,4-D) in liquid as well as in agar medium. Germination was induced by chilling but not by desiccation treatments. Epicotyl dormancy was overcome by placing the embryos with elongated radicles on medium with 6-benzyl-adenine (BA). Normally developed plantlets were transferred to soil, and acclimated in a greenhouse.

Key words: cork oak, germination, plant regeneration, *Quercus suber*, somatic embryogenesis, tissue culture.

Introduction

Cork oak (*Quercus suber* L.) is a forest species present in many countries of the Mediterranean basin, where it is exploited for cork production. Cork oak is mainly propagated by seed. However, seedling production from acorns is sometimes not advisable because of the high heterozygosity due to wind pollination. Therefore, the preservation of some characteristics can only be obtained by vegetative propagation. Nevertheless, conventional techniques, such as grafting or cutting, have proven particularly difficult for oaks, and this has encouraged us to look for alternative propagation methods.

Micropropagation of juvenile and adult material was reported previously (PARDOS, 1981; MANZANERA and PARDOS, 1990). However, a higher amount of explants can be produced by somatic embryogenesis.

Somatic embryogenesis has been little studied in this species (EL MAATAOUI and ESPAGNAC, 1987), and plant regeneration has not been achieved until now.

Material and Methods

Plant material

Samples were collected every two weeks during the period of fruit development, from June 25 until September 19, 1990. Pollination took place in May. Eight trees were selected for their good cork and fruit production in "La Herguijuela" (Cáceres, Spain). Fifty open pollinated acorns were taken per tree, per collection date. In June, only a few immature embryos were visible. In July, embryos were at the heartshaped phase. In August, the cotyledons grew and filled the ovule cavity. In September, the embryos were mature. Acorns were sterilized for 20 min with 2% NaOCl plus a few drops of Tween 20, followed by three rinses in sterile destilled water for 10 min each. Complete embryos were extracted from the ovule and cultured.

Culture medium

The basal culture medium comprised macronutrients of SOMMER et al. (1975) and micronutrients of MURASHIGE and SKOOG (1962), with the following additions: ascorbic acid (11.3 μM), nicotinic acid (8.1 μM), glutamine (3.4 mM), calcium pantothenate (4.2 μM), pyridoxine · HCl (4.9 μM) and thiamine · HCl (3 μM). Sucrose (87.6 mM) was used as carbon source. The following growth regulators were used: 6-benzyl-adenine (BA) and 2,4-dichlorophenoxyacetic

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