TRANSFORMATION OF SLASH PINE

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Abstract.--Genes responding to environmental stresses are being identified in Pinus and other woody plant species. The development of Pinus tissue culture systems are needed in order to: (1) characterize stress responsive genes in Pinus, and (2) to transform Pinus species with stress responsive characteristics. An embryogenic tissue culture system has been developed for slash pine (Pinus elliottii Engelm.) with the objective of understanding gene function as well as for developing technology for gene transfer and genetic transformation. Plantlets have been produced via embryogenesis. Foreign gene transient expression has been obtained in cell suspensions using a gas-driven particle bombardment procedure. A more stable, transformed callus has been obtained after bombardment with a gene providing resistance to the antibiotic, kanamycin. This progress in the development of transformation technology will help us understand the role(s) of stress-responsive genes in Pinus.

Keywords: Pinus elliottii Engelm., transformation, in vitro culture, embryogenesis, particle bombardment.

INTRODUCTION

Slash pine (Pinus elliottii Engelm.) is one of the hard yellow pines indigenous to the southeastern United States (Lohrey and Kossuth 1990). It is one of the two southern pines used for naval stores and is one of the most frequently planted timber species in North America (Lohrey and Kossuth 1990). It is favored by many forest managers because of its fast growth and excellent utility for pulp, lumber, and poles (Sheffield et al. 1983). It has the smallest native range of the four southern pines and grows naturally from South Carolina south to central Florida and west to Louisiana. It has been established by planting as far north as Tennessee and as far west as eastern Texas where it now seeds naturally (Lohrey and Kossuth 1990). In the 3 decades prior to 1980, the slash pine ecosystem increased by 22% and peaked in the late 1970s and early 1980s (Sheffield et al. 1983). Today, the rate of planting outside the natural range has slowed compared to the 1950 to 1970 period.

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Slash pine seed source planting trials outside the natural range indicate that it is not well adapted to extreme northern and western environment stresses such as low temperature and extreme drought (Switzer 1959; Snyder et al. 1967). Furthermore, slash pine is most susceptible to the fungal fusiform rust disease (Blakeslee 1983). Many trees are killed due to these stresses, and because of this there is a much lower amount of slash pine acreage planted each year compared to other Pinus species. For example, out of 27,000 acres of Pinus plantings administered by the Texas Forest Service in 1992, less than 100 acres were planted with slash pine (Barber 1993).

Much research has been conducted relative to identifying genetic variation in conifers in response to disease (Wright 1976; Zobel and Talbert 1984) and drought (Van Buijtenen et al. 1976; Pallardy 1981; Larsen 1981; Newton et al. 1992). Furthermore, many of these responses can be characterized at the molecular and gene level (Newton et al. 1991; Tauer et al. 1992; Funkhouser et al. 1993), and genetic transformation has been suggested as one of the alternatives for providing tolerance to both disease (Lamb et al. 1992) and drought (Newton et al. 1991; Funkhouser et al. 1993). For example, genes encoding a chitinase or a ribosome-inactivating protein (Lamb et al. 1992) could be tested for their conference of protection against fusiform fungal attack. In our own laboratories, we have identified a methyltransferase gene from Pinus taeda (*J.* Cairney, S. Chang, R. Newton, nonpublished) which is homologous to a parsley gene (Schmitt et al. 1991), and whose over-expression could modify lignin deposition (Sederoff et al. 1991). Lignin deposition is one of several multigenic defenses known for protection against microbial pathogens and it is proposed as a strategy for manipulation (Lamb et al. 1992).

With new biotechnologies being developed for plant transformation, it appears that they could be used on behalf of slash pine, i.e. to modify the species' tolerance to either drought and/or disease stress. Two technologies that are rapidly advancing in development for this purpose are: (l) gene transfer, and (2) in vitro culture. Recent advances in genetic transformation via the biolistic process represent a new approach to the problem of how to deliver DNA into intact conifer cells. Use of microprojectile-mediated DNA transfer has been reported for embryogenic cells of Picea glauca (Ellis et al. 1991; 1993), P. abies (Newton et al. 1992; Yibrah and Clapham 1990), P. mariana (Duchesne and Charest 1991), and the cotyledons of Pinus taeda (Stomp et al. 1990). In addition to DNA delivery capabilities, in vitro culture technologies with conifers are also advancing, particularly those associated with embryogenesis (Tautorus et al. 1991; Gupta et al. 1993).

Embryogenesis is the process of embryo formation from the zygote. The term somatic (or asexual) embryogenesis is applied when the embryo originates from cells that are not the product of gametic fusion. Somatic embryogenesis in conifers has progressed since the first report in 1985 (Hakman et al. 1985). Currently, the development of somatic embryos (SEs) on solid media has been reported in Norway spruce (Picea abies (L.) Karst) (Hakman et al. 1985; Krogstrup 1986; von Arnold and Hakman 1986) sugar pine (Pinus lambertiana Dougl.) (Gupta and Durzan 1986), slash pine (Pinus elliottii Engelm.) (Jain et al. 1989), loblolly pine (Pinus taeda L.) (Becwar et al. 1990; Gupta and Durzan 1987), white spruce (Picea glauca (Moench) Voss) (Hakman and Fowke 1987), and black spruce (Picea mariana (Mill.) B.S.P.) (Hakman and Fowke 1987). Spruces, larches, firs, and pines have been the focus of many studies,

and many have yielded successful results although plantlet recovery is often poor (Hakman and von Arnold 1985; Atree et al. 1990; Tremblay 1990).

Research in our laboratories has focused on the following three objectives: (1) identifying and characterizing stress-induced genes in Pinus, (2) developing suitable in vitro regenerations systems in Pinus, and (3) developing suitable DNA transfer protocols for Pinus transformation. In this paper, we report a successful regeneration system for Pinus elliottii, and our progress in regard to tissue transformation in this species.

MATERIALS AND METHODS

Plant materials

Weekly collections of green seed cones from one slash pine orchard tree (S2PC1) were made from an open-pollinated orchard of the Texas Forest Service in Magnolia Springs, Texas during the time interval of June 11, 1991 to July 2, 1991. The cones were prepared as described by Marek-Swize et al. (1994).

Initiation and maintenance of embryogenic callus

Immature zygotic embryos were aseptically excised from the seeds under a dissecting microscope. To initiate calli, explants were plated on two variations of a Douglas Fir Cotyledon Revised media (DCR) (Gupta and Durzan 1986) solidified with 1% gelrite as described by Marek-Swize et al. (1994). After 4-6 weeks when induction had been accomplished, all embryogenic calli were placed on a modified DCR-F media (Finer et al. 1989) with growth regulator concentrations reduced to one-fifth proportions (2,4-D, 1.8µM; BA, 0.9µM) and sucrose levels elevated to 30g/L (DCR-F/5). Explant evaluations were made biweekly for their potential to form embryogenic calli, and they were subcultured biweekly. After several passages, the DCR-F hormone levels were reduced to one-tenth of their original levels (DCR-F/10) for long term maintenance and proliferation.

Somatic embryo (SE) maturation

Because prior protocols associated with slash pine SE maturation (Jain et al. 1989) were not successful, the established embryogenic calli were subjected to the following subculture protocol: embryogenic calli with Stage 1 SEs were transferred from DCR-F/10 medium to DCR medium containing activated charcoal (1%, w/v) and sucrose (30 g/L) for 1 week, followed by subculture on DCR medium containing sucrose (30 g/L), ABA (10µM), and BA (1µM) for 4-5 weeks, and a final subculture on DCR medium containing sucrose (30 g/L).

For maturation to plantlets, Stage 3 SEs were immersed upside down in a DCR-agar medium and culture plates were inverted according to procedures of Becwar et al. (1989) at 25°C and light intensity of 250 µ molm $^{-2}$ sec $^{-1}$ until roots developed. The rooted plantlets were transferred to tubes containing a DCR medium with sucrose (30g/L) for root elongation until they reached a size of 2-3 cm. They were transferred to 8 cm pots containing peat, perlite and vermiculite (1:1:2) and allowed to acclimate in plastic bags for 4 weeks before transferring to the greenhouse.

Cell Suspensions

Embryogenic calli were initiated from immature zygotic embryos (Marek et al., 1994), and subcultured every 2 weeks in darkness at 25 C. Embryogenic cell suspensions were established from embryogenic calli pieces introduced into liquid DCR medium. They were cultured in 250 ml flasks containing 50 ml of medium in darkness at 25 C on a rotary shaker at 150 rpm. They were subcultured every 2 weeks. Two hundred mg of tissue were spread evenly on a filter disk in the form of a thin, circular layer (diam=5.5 cm) using a Buchner funnel with vacuum. The disks were placed on the solid medium before and after bombardment. In some cases the solid medium contained ABA (10µM).

Plasmids

The Dc8/GUS plasmid was obtained from Z. R. Sung, Univ. of Calif., Berkeley, and contains the GUS structural gene fused between the ABA-responsive, 1.5 kb 5' upstream region of the Dc8 gene isolated from carrot (Hatzopoulos et al. 1990) and the nopaline synthase polyadenylation sequence, cloned into a pUC plasmid, total size 6.6 kb. The Dc3/GUS plasmid was obtained from T. L. Thomas, Texas A&M Univ., and contains the GUS structural gene fused to a 1.5-kb 5' upstream element of the carrot promoter, Dc3 (Seffens et al. 1990). The 70S/GUS plasmid (pJIT65) contains a GUS structural *gene,* a double tandem 35S promoter and a 35S polyadenylation sequence (Guerineau et al. 1990) cloned into a pUC plasmid, total size 5.6 kb. The Em/GUS plasmid (pBMll3Kp) was obtained from R. S. Quatrano, Univ. N. C., and contains the ABA-inducible promoter from the Em gene of wheat (Litts et al. 1987) which is linked in translation fusion to GUS and a 3' flanking region from CaMV 35S cloned into the pUC derived plasmid (pBM113Kp). For kanamycin resistance and stable transformation, the plasmid pBl01.5 constructed in our laboratory (S. Chang and N. Dong) containing nos/NPTII and Dc8/GUS, was used.

Preparation of Particles

DNA was precipitated onto gold particles using the $CaCl_2$ precipitation procedure of Klein et al. (1988); 10 µg of supercoiled plasmid DNA added to 3 mg gold particles [diam = l.6 µm (BioRad)] were finally suspended in 50 µl ethanol. Aliquots of 10 µl were pipetted onto the macrocarrier.

Bombardment

The Dupont Biolistic™ particle delivery system (PDS-1000) was modified with a helium gas driven system. Each filter disk was bombarded while on the medium. To optimize bombardment conditions, the filter disks were placed at a distance of 74 mm from the stopping screen, under a chamber vacuum of 28.5 inch Hg, and bombarded with a rupture disc of 650 psi. After bombardment, the filter paper disk on the solid medium was incubated at 25 C for 2 d.

GUS Assay

ß-glucuronidase expression was histochemically assayed with 5-bromo-4-chloro-3-indolyl glucuronide (X-GLUC) (Jefferson 1987) 2 d after bombardment.

Five hundred µl of X-GLUC were applied to tissues on each disk contained in petri dishes and maintained at 37°C in darkness for another 1 d. Blue spots were counted under a dissecting microscope and the mean number and standard error were computed for each treatment. Spot number is correlated with the fluorimetric enzyme activity assay (Ellis et al. 1991).

Callus Growth Measurement

With stable transformation studies, callus growth was quantified by measuring their diameters from photographs taken at the beginning of the experiment and 20 days later.

RESULTS AND DISCUSSION

Slash Pine Embryogenesis

The time of cone collection spanned over a 4 week period. These explants produced embryogenic calli at an overall rate of 18% (Table 1). Within 4-6 weeks on induction media, callus development had begun on the explants. Calli appeared to develop from explant suspensor cells at the base of the developing embryo head. Calli derived from the explants proliferated rapidly and maintained their embryogenic potential with Stage 1 SEs. For somatic embryo (SE) development and maturation, embryogenic calli with Stage 1 SEs were subcultured. This subculturing protocol produced not only a high number of Stage 3 SEs, but also sixty-five Stage 4 plantlets (Table 1). Presently, nineteen plantlets have been placed in pots.

Table 1. Frequency of embryogenic calli and number of Stage 3 somatic embryos and Stage 4 plantlets of *Pinus elliottii.*

Frequency of embryogenic calli/explant	Number of Stage 3 Somatic Embryos	Number of Stage 4 Plantlets
85/454	215	65

In 1988, researchers in our laboratory were the first to establish embryogenic calli from immature zygotic embryos of slash pine (Jain et al. 1989), and we reported for the first time the maturation of slash pine embryogenic calli into plantlets (Marek-Swize et al. 1994). Along with loblolly pine *(Pinus taeda)* (Becwar et al. 1990; Gupta and Durzan 1987), this is the second commercially important *Pinus* species in the Southeastern United States for which this has been accomplished.

Immature zygotic embryos have served as an excellent explant source for SE production in a variety of conifer species. The optimum stage of immature zygotic embryo development as an explant source for initiation of embryogenic tissue in *Picea* species is postcotyledonary (Hakman et al. 1985; Hakman and Fowke 1987) while that for *Pinus* species (Finer et al. 1989; Gupta and Durzan 1987; Jain et al. 1989) including slash pine is precotyledonary. Immature

(Hakman et al. 1985; von Arnold and Eriksson 1981) and mature (von Arnold 1987; von Arnold and Hakman 1986; Gupta and Durzan 1986) zygotic embryos appear to be the best explants for initiating embryogenic calli since they have greater degrees of competency than other tissue types. Conifer embryogenic calli SEs can mature and develop further when placed on auxin-free solid or liquid media (Gupta et al. 1993). In Norway spruce, auxin appears to be required for differentiation of new SEs, and cytokinin is required for retaining somatic embryo organization (Bellarosa et al. 1992).

Abscisic acid also has been shown to enhance SE maturation (Gupta et al. 1993; Durzan and Gupta 1987; von Arnold and Hakman 1988). In addition to an auxin-free ABA-containing medium, enhanced SE maturation also results from an increased osmolality, particularly in early stages of embryo development (Gupta et al. 1993). Osmotica such as sorbitol, mannitol and myo-inositol in the media reduce its osmotic potential and subject SEs to water stress. However, the elevated levels of sucrose that were maintained in all subculture media in our study, may have had an osmotic function in enhancing maturation. With sucrose concentrations ranging from 10 to 50 g/L (30 g/L being optimal), Norway spruce SE maturation was enhanced (von Arnold and Hakman 1986; Hakman and von Arnold 1988).

Slash Pine Transformation

Microprojectile DNA delivery has been demonstrated to be a suitable method for conifer species (Newton et al. 1992; Charest et al. 1993; Stomp et al. 1991). With this technology, we wanted to optimize the delivery system and to assess several promoter sequences as to their capability for driving GUS gene expression in transformed cell suspensions of slash pine. Five promoter sequences fused to the GUS gene were assessed by counting the number of blue spots/disk 48 hr after bombarding with DNA (Table 2). For comparison, the CaMV 35S promoter is often used as a standard. Its performance was compared with the tandem repeat of 35S (CaMV 70S) and the abscisic acid (ABA)-regulated promoters (Dc3, Dc8, and Em). The latter three were selected because: (1) many gene responses to stress are mediated by ABA (Skriver and Mundy 1990; Newton et al. 1991), and (2) somatic embryo maturation in slash pine (Marek-Swize et al. 1994) and other conifers (Gupta et al. 1993) is mediated by ABA. The promoter, Dc8, was also chosen for study because it is not inducible in nonembryonic cells by ABA (Hatzopolous et al. 1990).

The tandem repeat, CaMV 70S promoter, appeared to be as effective as CaMV 35S in driving GUS expression in slash pine cells suspensions, while the Dc8 promoter showed the least expression (Table 2). A maximum number of 1845 spots/disk were obtained with the Dc3 promoter with one bombardment, and a mean of 764 was obtained with 10 bombardments (Table 2). On the average, the Em promoter-driven GUS expression was less than that driven by Dc3, but was similar to that driven by both DC3 and CaMV 70S (Table 2). Although the number of bombardments was limited, the Dc8 promoter was more responsive to the ABA treatment than was Dc3 (Table 3). Dc8-driven GUS expression was also enhanced by ABA in transformed tissues of Norway spruce (Newton et al. 1992).

Table 2. Promoter-driven GUS expression in embryogenic cell suspensions of Pinus elliottii.

Promoter	Number in Sample (n)	Number of Blue Spots/Disk Mean + S.D	Maximum
35S	10	433±264	979
70S	3	642±195	832
Dc3	10	764±479	1845
Dc8	10	176±63	268
Em	3	694±140	852

Table 3. Effect of abscisic acid (ABA) on promoter-driven GUS expression in embryogenic cell suspensions of Pinus elliottii.

Promoter	Number in Sample (n)	Mean (±SD) Number of Blue Spots/Disk Control[a]	ABA[b]
35S	3, 3	352±66	339±49
Dc3	3, 3	591±102	374±110
Dc8	3, 3	151±23	296±29

[a] 2 days on media without ABA
[b] 2 days on media with ABA (10 µM)

These data indicate that there is a high level of GUS expression in slash pine embryogenic cells, but the challenge still remained to sustain the expression as well as regenerating plants from those cells which stably incorporated the introduced genes into their genome. Transformed cell selection with an introduced kanamycin-resistance gene (nos/NPTII) which subsequently leads to stable transformation has been accomplished with white spruce (Ellis et al. 1993). Sublethal kanamycin levels in the media was used to select for transformed cells. We have utilized a similar procedure with nos/NPTII and kanamycin resistance introduced into slash pine cell suspensions.

Slash pine cell suspensions were bombarded with nos/NPTII+Dc8/GUS and converted back to callus. The calli were then subjected to two selection regimes: (1) 45 days on kanamycin media with a concentration of 20 pg/ml, and (2) 30 days on kanamycin with a concentration of 50 µg/ml . Calli derived from non-bombarded suspension cells (Control) and from the bombarded doubly-

selected calli (Bombarded) were then placed on a solid nutrient media containing kanamycin at four different concentrations (20, 40, 80 and 160 pg/ml). Callus diameter was measured at the time it was placed on the medium and after 20 days on the same media. The diameter of control calli decreased 0.3 to 0.7 mm on kanamycin media (Table 4). On the other hand, calli derived from bombarded cells increased in diameter on all of the kanamycin media (Table 4). These data indicate that the calli derived from suspension cells bombarded with nos/NPTII have been transformed and are resistant to kanamycin. These calli are still embryogenic and are being subjected to maturation treatments in order to derive transformed somatic embryos.

Table 4. Effect of kanamycin on growth of calli derived from cells with or without bombardment with nos/NPTII+Dc8/GUS and after 20 days on kanamycin media.

	Mean Callus Diameter (mm)[a]					
Kanamycin	Control			Bombarded		
Conc. (µg/m1)	0 days	20 days	Growth	0 days	20 days	Growth
20	2.7	2.3	-0.4	2.2	3.9	+1.7
40	2.7	2.0	-0.7	2.3	3.2	+0.9
80	2.4	2.0	-0.4	1.9	2.5	+0.6
160	2.4	2.1	-0.3	2.2	2.3	+0.1

[a] n = 24

With white spruce callus, the ability to form embryogenic callus at a high frequency was superimposed on the ability to suppress, yet not kill the tissue, during selection on kanamycin media (Ellis et al. 1993). The use of sublethal kanamycin levels (5 pg/ml) suppressed but did not kill non-transformed cells, while allowing transformed cells to divide (Ellis et al. 1993). The sublethal levels of kanamycin in our study with slash pine appears to be about 20 µg/ml (Table 4), and the transformed calli are still embryogenic. Transformed calli from Norway spruce SEs did not retain embryogenic potential when selected with 10 pg/ml kanamycin (Robertson et al. 1992).

CONCLUSIONS

Embryogenic callus of slash pine was initiated with auxin and cytokinin using immature zygotic embryos as explants. The callus was advanced toward SE maturation resulting in production of plantlets. This is the second commercial species of pine in the southern U. S. in which SEs have been produced. Cell suspensions of these embryogenic calli were placed on filter paper and bombarded with DNA plasmids coated on gold particles. Five different promoters fused to GUS were transiently expressed in slash pine cells, with the Dc3 promoter from carrot providing the greatest GUS driven expression. Calli stably transformed with nos/NPTII resulted from selection on media containing kanamycin. With an available, rapidly growing, conifer culture system, coupled with a suitable DNA delivery system, more progress in biotechnology research can be achieved leading to eventual genetic transformation and understanding gene expression in pines.

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