XIII Genetic Transformation of *Pinus palustris* (Longleaf Pine)

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1 Introduction

Longleaf pine (*Pinus palustris* Mill.) is an important softwood species in the southeast United States. In presettlement times, this species occupied extensive, pure stands throughout the Atlantic and Gulf Coastal Plains from southeastern Virginia to eastern Texas, as well as south throughout the northern two-thirds of Florida. Its range also includes the Piedmont Ridge and Valley, and Mountain Provinces of Alabama and Georgia (Burns and Honkala 1990). Historically, longleaf pine was the premier timber and naval stores tree, a fact which resulted in its virtual disappearance from extensive regions. Its value as a timber species remains high; it shows excellent form and good wood qualities, as well as resistance to fusiform rust, the most damaging disease of southern US three-needle pines. An aspect of longleaf pine which negatively affects its relative reforestation value is its grass stage, during which its first 5 years of growth remains essentially limited to root development. This stage is also characteristically expressed for several years by adventitious micropropagules generated in vitro, although a few genotypes have shown precocious and rapid shoot elongation (AM. Diner, unpubl.). Notwithstanding the obstacles to seedling growth presented by the grass stage, however, the value of this tree has compelled widespread reforestation efforts.

Current perspectives for value-added longleaf pine genetic transformants relate to both disease resistance and early shoot growth. The major microbial disease of this species is brown-spot needle blight *(Scirrhia acicola)*, which causes severe defoliation and death to grass-stage seedlings. Other commercially important microbial diseases include pitch canker *(Fusarium moniliforme* var. *sub&tans),* annosus root rot *(Heterobasidion annosum)*, and cone rust *(Cronartium strobilinum)*. Longleaf pine suffers attack by a variety of defoliating insects, including both adult (*Colaspis pini* Barber) and larval [(*Neodiprion lecontei* (Fitch); *Dendroctonus terebrans* (Oliv.); *Hylobius pales* (Hbst.); *Pachylobius picivorus* (Germar); *Dioryctria* spp.; Laspeyresia spp.)] insect forms. Because vector systems exist for plant transformation to such as chitinase and BT toxin syntheses, opportunities for transformation of longleaf

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pine for pest resistance are potentially available. Indeed, the whole-tree *Larix* transformant has been regenerated expressing BT toxin synthesis (Shin et al. 1994), suggesting that *Pinus* may be similarly transformed. Moreover because shoot growth restriction in grass-stage plants is a probable result of endogenous plant growth regulator (PGR) control, transformation of tissues for modified PGR synthesis, followed by regeneration of the plant, may provide early shoot elongation in the whole-tree transformant.

Longleaf pine shows great genetic variation in those traits affecting survival, growth, and disease resistance (Schmidtling and White 1989), suggesting its useful candidacy for clonal propagation. Rooting of cuttings is possible, but unreliable. Grafting is now the most common method used to establish seed orchards. However, methods are available for longleaf pine somatic embryogenesis (Nagmani et al. 1993), which allow opportunity for genetic manipulation and regeneration of the transformed regenerant. Since tissues of several species of pines have been transformed using biolistics, and this author was successful in regenerating *Larix* whole trees from Agrobacterium-induced transformants (Diner and Karnosky 1989; Huang et al. 1991), both procedures for genetic transformation were undertaken using longleaf pine.

2 Genetic Transformation

2.1 Agrobacteria

Attempts to genetically transform longleaf pine were carried out, employing several strains of agrobacteria (Diner and Soliman 1993). Methods developed (Diner and Karnosky 1987; Huang et al. 1991) for transformation and concomitant whole-tree regeneration of *Larix* using agrobacteria were used with armed strains of *Agrobacterium* inoculated to barely germinated seedlings (germlings) of longleaf pine showing 1- to 5-mm roots. The microbial inoculum consisted of 1–2 µl of a dense agglomeration of log-growth cells, scraped from a lawn on agar-solidified LB medium (Huang et al. 1991). This inoculum was applied to the narrow cavity of 1-3-mm longitudinal wounds made to the cotyledons, hypocotyl apices, and roots. The use of a heavy inoculum of the vector characteristically supports high transformation frequency (Lippincott and Lippincott 1975). Strains of *Agrobacterium* used included *Agrobacterium rhizogenes* American Type Culture Collection (ATCC) strains 11325, 31798, and *15834,* as well as *A. rhizogenes* A4 and A4pARC8 (Simpson et al. 1986). Strain 11325 was selected due to its having uniquely and reliably produced tumors of normal shoot phenotype in *Larix* (Diner and Karnosky 1987; Huang et al. 1991), thus allowing (with subsequent adventitious root initiation) whole-tree regeneration of the transformant. Strains A4 and A4pARC8 were highly root-tumorigenic in *Larix* (Huang et al. 1991). Strains of *A. tumefaciens* used included Bo542 and A281 (Hood et al. 1985), A208 (provided by Wayne

Barnes, Dept. of Biochemistry, George Washington University Medical School, St. Louis, MO), and ATCC 15955. The former three strains were moderately to highly gall-tumorigenic in *Larix* (Huang et al. 1991). Each bacterial strain was inoculated to at least 150 germlings, with at least 50 germlings each inoculated at one site only. Once inoculated, the germlings were placed in agar-solidified Gresshoff and Doy medium (Mehra-Palta et al. 1978) in Petri plates, which were then sealed with Parafilm and incubated for 30 days at 20 °C under 80 $\mu E/m^2/s$ cool-white fluorescent illumination. Controls included both wounded-uninoculated and intact germlings. Seedlings were appraised at 30 days for presence and characteristics of any anomalous tissue and growth suggesting tumorigenesis by the *Agrobacterium* vector employed.

2.2 Microprojectile Bombardment

Biolistic transformation of longleaf pine embryogenic masses was attempted using 2-year-old cultures initiated and maintained as described (Nagmani et al. 1993). Three months prior to bombardment, cultures were transferred to, then maintained on a pH. 5.8, 0.65% agar (Sigma Chem. Co.)-solidified Brown and Lawrence medium (1968) containing 2.5% sucrose, and modified to contain glutamine (10 μM) as the sole source of amino nitrogen. The medium was supplemented with 2,4-D (8 μM) and BAP (4 μM). All cultures were dark-incubated at 20–22 °C, and subcultured every 3--4 weeks to freshly prepared medium. Five to 7 days prior to bombardment, the embryogenic culture of each species was transferred to fresh medium, such that each subculture occupied an approximate circular area of 2-3 cm diam. in the center of each plated medium. Six replicate cultures were prepared for each species, of which one was to be an unbombarded control.

Procedures used for microprojectile preparation and coating with DNA have been described (Heiser 1992). Each culture was bombarded once with 1-μg gold particles onto which had been precipitated the construct pAMTGUS25 (Diner et al., in press); (provided by Amitava Mitra, University of Nebraska, Lincoln), containing the *Chlorella* virus adenine methyltransferase promoter gene (Mitra and Higgins 1994) linked to the GUS Reporter gene (Jefferson et al. 1987). Twenty-five μl (μg/μl) DNA was precipitated onto 3.6-μg gold particles. Initial bombardment employed a sample distance of approximately 6.3 cm. This was later changed to 5 cm, in order to increase particle pattern density, and thus potential transformation frequency. All bombardments were carried out using a rupture disk pressure of 1100 psi. The apparatus employed for bombardment was the Biolistic particle delivery system PDS-1000 (DuPont, Wilmington, DE). Following bombardment, Petri dishes containing the cultures were incubated in the dark at 20-22 “C. Cultures were sampled at 48 h and 7 days. Sample size was, approximately 0.3 mg/l. GUS activity was assayed as described (Jefferson et al. 1987).

2.3 Results and Discussion

Wound-inoculated cotyledons and roots necrotized within 1 week, as did wounded, uninoculated roots. Because very young roots of the size(s) employed here are fragile and commonly do not survive small wounds made inadvertently during manipulation, their failure to survive wound inoculation was not surprising. The fact that inoculated cotyledons did not survive may have been an additive effect of inoculating so small an organ with a dense population of growing bacteria which would inevitably prove tissue-necrotic in vitro. Cotyledons of 5- to 7-week-old intact seedlings of *Pinus halepensis* Mill. did not show a similar propensity to necrosis following wound inoculation using a 25G needle (Tzfira et al. 1996). However, these were 5- to 7-week-old seedlings, as opposed to the barely emerged longleaf pine seedlings described here, and which had been inoculated using a larger wound than that produced by a 25G needle. Thus, greater size, reduced fragility and a reduced proportion of seedling tissue damaged by wound inoculation may have influenced survival of seedling organs.

It is possible that 30-day cocultivation of longleaf pine with an agrobacterial inoculum may not have been definitive. Recorded tumor frequency involving any of the bacterial strains or vectors employed may have proven greater if longer periods (Tzfira et al. 1996) had been used. Pathological responses to inoculation are provided in Table 1.

Frequencies of longleaf pine tumorigenesis by strains A4 and A4pARC8 were lower than those reported for *Larix*, though tumor phenotype was consistent (Huang et al. 1991). Strain ATCC '15955 was tumorigenic on neither *P. palustris* (these data) nor *P. ponderosa* Laws. (Morris et al. 1989), though other conifer genera were susceptible (Morris et al. 1989). Strains Bo542 and A208 showed similar frequency of tumorigene:sis in *Larix deciduu* (Huang et al. 1991) and *P. palustris* (these data). However, tumors induced by strain A208 inoculation to longleaf pine were consistently of the hairy root morphology

Table 1. Longleaf pine pathological responses to *Agrobacterium* inoculation

Strain	Plasmid	Tumor formation frequency (%)	Tumor phenotype
A. rhizogenes			
A4	Wild Ri	13	Root
A4pARC8	Binary	19	Root
ATCC 11325	Wild Ri	0	-
ATCC 15834	Wild Ri	0	
ATCC 31798	Wild Ri	0	-
A. tumefaciens			
Bo542	Wild Ti	87	Crown gall
A208	Wild Ti	54	Root
A281	Wild Ti	21	Crown gall
ATCC 15955	Wild Ti	0	-

Values represent at least 50 replicates of each treatment shown.

(Fig. 1A), rather than the amorphous tumor growth reported in *Larix,* and characteristic of inoculation with this strain of *A. tumefaciens*. Unexpected or presumably uncharacteristic tumor morphologies induced by particular species, strains, or plasmids of agrobacteria are no4 rare, and may depend upon several factors relating to both the host and the bacterium (Ellis et al. 1994). Our objectives for plant transformation involve regeneration of the whole-tree transformant. Methods are not yet available for conifer regeneration from hairy root or gall-type tumors. Certainly, there are a great many agrobacterial strains, as well as vectors, and possible vector constructs. Notwithstanding, the somatic embryogenic systems in hand, coupled with biolistics, appear to potentially offer a more productive approach to genetic transformation and regeneration in *Pinus*.

All bombarded embryogenic cultures expressed GUS activity at 48h. While the controls did not. Bombardment at a target distance of 6.3 cm produced approximately eight impact/GUS expression sites per sample. Bombardment at 5 cm approximately doubled that effect, as measured up to the

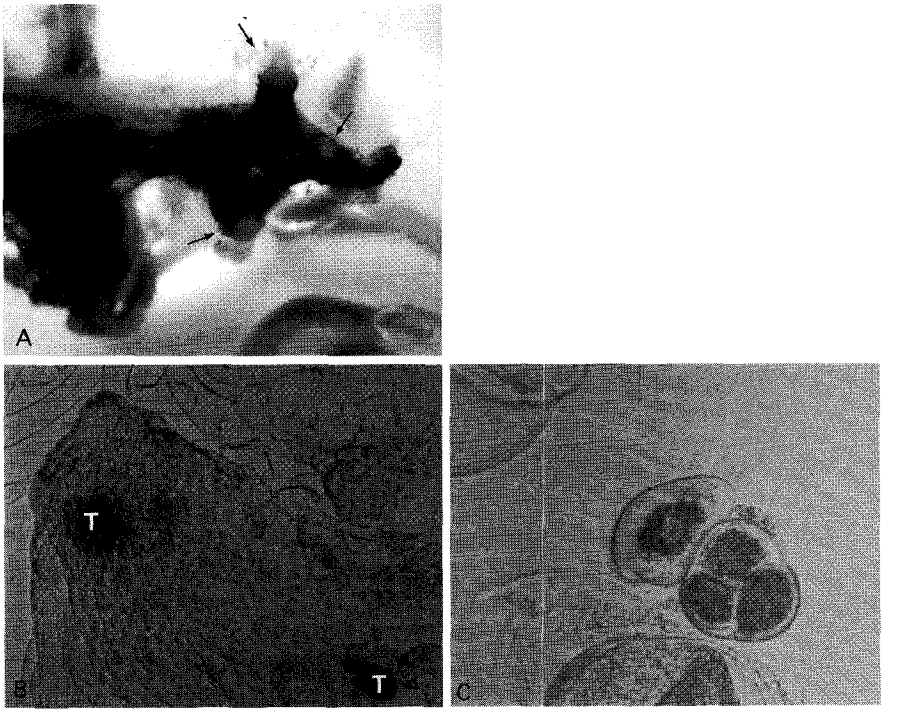

Fig. 1. A Several roots ***(arrows)*** emerging from a hypocotyl wound site on a longleaf pine germling inoculated with ***A.*** *tumefaciens* A208 (10x). **B** Two loci (*T*) expressing GUS on a bombarded longleaf pine somatic embryo. Areas adjacent ***(arrows)*** to the GUS expression sites show a diffuse and less intense blue color (400×). C Four clustered blue cells from a bombarded longleaf pine embryogenic mass. Three appear to have had a common GUS-expressing progenitor ***(1200x)***

maximum 40x magnification provided by the binocular dissecting photomicroscope used to examine the microcentrifuge tube content of bombarded tissue. However, greater resolution, later provided by the compound microscope employed for photomicroscopy of transformant cells in thin layer, showed both multiple GUS blue-colored impact sites on unit structures such as single embryos (Fig. 1B), and numerous isolated individual cells or small clumps thereof showing the same characteristic blue color. This was especially true of tissue bombarded at 5cm. Many of these GUS expression sites were often densely associated, rendering an accurate count difficult. The number of GUS-expressing cells might have been increased had a filter been used to support the embryogenic cells during bombardment, or had other microcarrier coating protocols been considered (Walter et al. 1994). Forty eight-hour assays showed GUS activity in single cells (Fig. 1C) as well as in small clusters of three to six cells. The latter may have resulted from mitotic events in the 48 h prior sampling to or may indeed have resulted from leakage. Cells in somatic embryo heads (Fig. 1B) and suspensors also expressed GUS. In the former case, where cells were densely packed, stain appeared either to have diffused into adjacent cells, or reacted with substrate which, itself, had diffused from the specifically bombarded/transformed cell. This phenomenon was also reported for bombarded pine cotyledons (Stomp et al. 1991). The promoter employed here was a 851-bp upstream region from an algal virus methyltransferase gene, heretofore shown effective for expression in transformants of a small number of both monocots and dicots (Mitra et al. 1994). No applications of this promoter to tree transformation have been reported, and, although our assays showed no GUS activity after 7 days, such a temporal loss of expression might be reduced through use of certain medium osmotica or of different promoters (Li et al. 1994; Martinussen et al. 1994). Other promoters are showing greater promise for long-term or stable expression in bombarded *Pinus* (Walter et al. 1994), such that totally transformed longleaf or other somatic embryos of pine species might be developed.

3 Summary and Conclusions

Tissues of longleaf pine germlings were genetically transformed using several individual wild strains of agrobacteria. With one exception, resultant tumor morphologies were those documented for the strains used. Transformation of the host pine was determined expressed by development of the tumor; Southern blot analyses were not performed. The strain (***A. rhizogenes*** ATCC 11325) responsible for shooty tumorigenesis in *Larix* proved innocuous in *P. palustris.* Thus, irrespective of tumor morphology, regeneration of the whole-tree transformant proved unlikely. Transformation of individual cells in somatic embryogenic masses was a reliable result of microprojectile bombardment. However, expression of the transforming marker was transient, possibly a result of the gene promoter used.

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