A GENOTYPE-INDEPENDENT METHOD FOR TRANSFORMATION OF PINES USING *AGROBACTERIUM*

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Forest products constitute the third most important cash crop in Texas after cotton and grain sorghum. Of the forest species, loblolly pine is the most important. The single most important factor blocking improvement of loblolly and other commercial pine genotypes through genetic engineering has been an inability to regenerate plants following transformation. To overcome this block in plant regeneration, we modified a direct shoot apex inoculation procedure using *Agrobacterium,* developed earlier for cotton and maize (Gould et al., 1991). Inoculation of a pre-formed shoot facilitates plant regeneration and makes the procedure genotype-independent. The potential for somaclonal variation caused by tissue de-differentiation in culture is reduced and total time in culture is minimized. The method exploits super-virulent *A. tumefaciens* (Hood et al., 1986;1990), transformation competent cells in the apical meristem and the native capacity of the shoot apex to regenerate into a complete plant. Using this method, we have recovered transgenic plants of loblolly pine and putative transgenic plants of afghan, radiata and Virginia pines. The evidence obtained for the genetic transformation of loblolly pine are: promoter-dependent GUS expression, PCR amplification of sequences unique to *nptll* in the DNA of regenerated plants, and high molecular weight DNA with homology to the transferred *uidA* (GUS) and *nptll* (kanamycin resistance) genes, characteristic of genomic incorporation.

Pinus taeda and other forest trees have only recently begun to be domesticated through selection and breeding. Significant improvement can be achieved through marker-aided selection and propagation of superior types. Specific and dramatic genetic gain may be possible through transformation with engineered genes; however, this achievement may be gained at the cost of genetic diversity since most plant transformation and regeneration procedures available are limited to regenerable genotypes. Although loblolly pine readily sustains genetic transformation by *A. tumefaciens* (Sederoff et al. 1986; Huang & Tauer, 1994) and particle acceleration methods, recovery of transformed plants from transformed tissue has been rare. To overcome these problems in plant regeneration from callus or embryogenesis following transformation, we used *A. tumefaciens* inoculation of the shoot apex (modified from Gould et al., 1991), and direct regeneration of plants from shoots. This approach exploits the evolved gene transfer mechanism of *Agrobacterium,* the dividing cell population in the apex that accepts *Agrobacterium* transformations, and the unique developmental characteristics of an apical meristem. The transformation method is genotype-independent and compatible with all pine species and existing tree improvement programs.

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Shoots from organogenesis in tissue culture or from germinating seedlings can be used in transformation, which permits application to elite and unique genotypes, seedlings identified through marker-aided selection, and can be adapted to clonal forestry.

Shoots of *Pinus taeda L.,* derived from cotyledonary explants were initiated as described (Jang & Tainter, 1991), separated and placed in shoot elongation medium (Meha et al., 1978; Mott & Amerson, 1981; Chang et al., 1991) until 0.5 to 1.0 cm in height. Shoot cultures were then stored at 4C for up to 3 months and then recultured on media containing a cytokinin 1 week prior to inoculation. Shoots isolated from germinated seedlings were also used. Shoots were inoculated with *A. tumefaciens* EHA101 (pGUS3) or EHA105(pSSLa). Both vectors carried *uidA* (GUS, beta-glucuronidase) and *nptll* (neomycin phospho-transferase II), the only difference being the promoter used with the GUS gene. The pGUS3 plasmid (Gould et al., 1991) contained the CaMV 35S promoter fused with GUS and was used in EHA101 (Hood et al., 1986: 1990), while pSSla.3 (Campbell et al., 1994), contained the larch Rubisco small subunit promoter fused with GUS and was used in EHA105. A single 4 week passage on a 50-75% lethal dose of 25 mg,/1 kanamycin was used. This value was chosen because it allowed escapes. The level of selection was intentionally kept low to insure survival of transformed shoots, since dividing cells in the shoot meristem are more sensitive to kanamycin than other cells. Approximately 10-20% of the inoculated shoots survived this treatment, although shoot mortality was not evident immediately. All shoots were recultured, and surviving shoots recovered and elongated to a 2 cm overall length in approximately 8-10 weeks. At this time, approximately 10% of inoculated shoots were ready for root induction; however, overall recovery of plants was low (1-3%) because of low rooting response. With development of a more efficient method for rooting loblolly shoots, recovery of plants can be greatly improved.

Elongating shoots and intact plants were assayed for GUS activity. Expression of GUS was promoter dependent: localized to phloem tissue with the CaMV 35S promoter (in pGUS3); restricted to emerging leaves flanking the shoot meristem with the Larch Rbcs promoter (in pSSLa). Southern DNA analyses showed transfer of both *uidA* (GUS) and *nptll* (kanamycin resistance) genes into high molecular weight DNA of recovered plants. PCR and Southern DNA analyses ruled out the possibility that the transferred genes were present in residual *Agrobacterium* contamination. We used the same transformation procedure with afghan, radiata and Virginia pines, and have recovered approximately 100 plants of afghan pine. These three species tolerate shoot meristem transformation well, root readily and can be useful in generating large numbers of transgenic plants for testing effectiveness of many genes and promoter sequences intended for use in loblolly or other pines.

In summary, we have used an *Agrobacterium* and shoot apex transformation method with loblolly pine and have recovered transgenic plants. Genetic fidelity is most closely maintained in the meristems of plants, and plant regeneration from isolated shoots and cuttings is straight-forward and simple. Isolated shoots are inoculated with a hyper-virulent strain of *Agrobacterium tumefaciens,* subjected to selection and generated directly into plants which makes the process genotype-independent. Tissues do not pass through a dedifferentiation

step to callus, and plant regeneration is not dependent on shoot organogenesis, somatic embryogenesis or limited to regenerable genotypes.

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