A Modified Mercuric Ion Reductase Gene May Offer An Alternative To Antibiotic Selection For Production Of Transgenic Southern Pines

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Growing public concern regarding the use of antibiotic resistance genes as selectable markers for transformation of agronomic and forest species has prompted research aimed at developing alternative selectable markers. Heavy metal resistance genes, which our labs have been investigating for their potential application for phytoremediation, may provide one such alternative, and for some groups of plants may actually prove to be more efficient selectable markers than antibiotic resistance genes. One such group is the genus *Pinus*. Despite great progress in the area of in conifer transformation, there have been only a few published reports of production of transgenic pines. The first reports of regeneration of transgenic pines were those of Walter and co-workers (Walter and Smith 1977, Walter et al. 1998), who used biolistics to engineer radiata pine (*Pinus radiata*) with marker genes. More recently, the same group reported the production of transgenic radiata pine engineered with the *bar* gene, which confers resistance to the herbicide phosphinothricin (Walter et al. 1997, Bishop-Hurley et al. 2001). Levee et al. (1999) reported production of transgenic eastern white pine (*Pinus strobus*) via *Agrobacterium*-mediated gene transfer. To date, however, there have been no publications reporting the transformation of the top commercial southeastern U.S. species, loblolly pine (*Pinus taeda*) and slash pine (*Pinus elliottii*). One problem potentially limiting the ability to regenerate transgenic pines may be the use of antibiotics and antibiotic resistance genes for selection of transformed cells. In both *P. radiata* and *P. strobus*, several weeks of selection on antibiotic-supplemented medium were required before resistant colonies of cells were observed. In addition, frequencies of transclones per gram of bombarded or inoculated embryogenic pine material were relatively low compared to those reported for angiosperm species. In the research reported here, we tested a modified bacterial mercuric ion reductase gene (*merA*; Rugh et al. 1996, 1998) and selection on mercuric ion-supplemented medium for their ability to provide selection of transformed slash and loblolly pine cells.

Embryogenic slash and loblolly pine cultures were initiated during the summer of 1999, and again during summer, 2000, from seeds collected from trees growing at the Georgia Forestry Commission's Flint River nursery and seed orchard near Byromville, Georgia. Seeds were dissected aseptically and megagametophytes with embryos were explanted onto 1/2P6 medium (Teasdale 1986) with 3 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.5 mg/l benzylaminopurine (BA). Following extrusion from the megagametophytes, embryogenic material was transferred to EDM6 medium (Smith 1996; Walter et al. 1998) for proliferation. Cultures were maintained by transfer to fresh EDM6 every 10 days.

For the first round of transformation experiments with *merA*, two slash pine and one loblolly pine embryogenic culture initiated in 1999 were bombarded with the vector pAL77, which carried the *merA9* gene driven by a maize ubiquitin promoter, using the Bio-Rad

PDS1000/He biolistics device. Following 5 days of recovery on EDM6 medium, bombarded cells were transferred to EDM6 medium supplemented with 15 µM $HgCl_2$. Colonies putatively resistant to mercuric ion began to arise within 1 week on this selection medium. These colonies were subsequently transferred to EDM6 medium with 30 µM HgCl2 as a secondary screen. A subsample of cultures that continued growth on 30 µM HgCl2 were assayed for the presence of the *merA* gene using PCR, and most produced the expected signal. However, in some cases a PCR signal was detected that did not correspond to the *merA* gene when a blot made from the gel was probed with radiolabeled *merA*. Overall, over 100 mercuric ion-resistant slash pine and loblolly pine embryogenic cultures were produced. However, probably due to the fact the cultures were over a year old by the time they were put into the embryo production protocol, no germinable somatic embryos were produced from the transclones produced by this round of bombardments.

The second round of bombardments was conducted using 20 embryogenic slash pine and 4 embryogenic loblolly pine cultures initiated in 2000. Transformation protocols and the vector employed were the same as used with the 1999 cultures. However, since our goal was to obtain germinable transgenic somatic embryos, bombardments were conducted on the newly initiated embryogenic cultures as soon as they had produced a few grams of material. An average of almost 13 mercuric ion resistant colonies per gram of bombarded tissue was obtained for slash pine, but only about 1 mercuric ion resistant colonies per gram of loblolly pine tissue was achieved. For slash pine, about 44 percent of the colonies that arose on 15 µM HgCl2 continued to proliferate following transfer to 30 µM HgCl2, while the figure for loblolly pine was about 37 percent. While PCR assays for the presence of the merA gene are continuing, to date, 25 out of 32 of the mercuric ion resistant slash pine colonies have tested positive for the presence of the *merA* gene. Several of these putative transgenic lines have produced embryos, and currently embryos from 7 of these lines have germinated.

The results summarized here indicate that the mercuric ion reductase gene and mercuric ion selection may provide a useful alternative to selection with antibiotic or herbicide resistance genes. Mercuric ion-resistant pine colonies appear to arise more rapidly than antibiotic-resistant colonies and perhaps at a higher frequency as well. The use of *merA* as a selectable marker has been patented by the University of Georgia Research Foundation. It should be kept in mind, however, that the use of *merA* and mercuric chloride as a selection system has its own potential drawbacks. First, ionic mercury is highly toxic and must be handled with appropriate safety equipment and thorough training. Secondly, previous reports of some angiosperm species transformed with *merA* have indicated that expression of the *merA* transgene maybe associated with some physiological problems in the transgenic plants (Rugh et al. 1996). Since free-living *merA*-transformed pine somatic seedlings have yet to be regenerated, whether or not a similar problem may occur with *merA*-transformed pines remains unknown at this point. Nevertheless, we believe our preliminary results show promise for the use of heavy metal resistance for selection in the production of transgenic southern pines.

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