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INFORMATION LEARNED FROM THE GENETIC ENGINEERING OF RECALCITRANT MONOCOT SPECIES: APPLICABILITY TO PROBLEMS IN FOREST BIOTECHNOLOGY

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ABSTRACT

Technologies in forest molecular biology and tissue culture, when coupled with classical techniques, could play an increasingly important role in the solutions to challenges in forestry and the processing of forest products. However, significant challenges exist in the genetic engineering of forest species. New insights into these problems can be gained by studying those encountered with other recalcitrant species, such as cereals, since many issues are the same, lack of reproducible and efficient transformation systems, identification of reliable selection schemes, problems with gene instability and potentially somaclonal variation. Improvements in culturing, bombardment and selection conditions were necessary in order to move transformation methods for model cereal varieties into methods effective for commercial cultivars. In addition, plants deriving from tissue culture frequently accumulate heritable genetic changes leading to negative field performance which requires backcrossing to wild-type varieties. Recent efforts have focused on targeting apical meristems and immature embryos attempting to minimize the mutagenic effects of *in vitro* culture. Attempts to develop improved methods for gene delivery. In summary, although transformation of a given species might be possible, there are challenges remaining in order to insure that genetic engineering technologies are useful for producing commercial germplasm.

INTRODUCTION

In the context of classical breeding the application of molecular genetic and tissue culture methods to commercially important trees species could play an increasingly important role in solving some fundamental challenges that face the forest products industries. This could include increasing yields by improving agronomic traits, such as enhancing pest and stress (cold and drought) resistance and herbicide resistance for forest planting. These methods could also be used to improve wood quality by controlling fiber length, microfibril angle, specific gravity, and altering lignin types/extractability to add value to tree fibers.

Despite this promise of biotechnological methods, significant problems still remain for introducing and expressing foreign genes in commercially important forest tree species. One way in which to gain new insights and identify new approaches to solve these problems is by learning from studies being carried out with other recalcitrant species, such as the cereals. Many of the issues and areas of biotechnological research in cereals are the same as those for forest trees. These include:

- Lack of reproducible and efficient transformation systems
- Identification of reliable schemes to select for transformed tissues
- Potential somatic mutation and epigenetic changes introduced during *in vitro* culture
- Transgene and transgene expression instability

Because these factors impact the practical utility of the technology, much work in the Lemaux laboratory focuses on developing strategies that minimize the negative factors needed to be able to utilize these technologies for agronomic

improvement of commercial cereal cultivars.

Applying Transformation Technologies Developed with Model Cultivars to Commercial Cultivars of Barley

By modifying previously reported methodologies for the transformation of rice (Christou, P., Ford, T.L., Kofron, M, 1992) and maize (Gordon-Kamm et al., 1990, The Plant Cell:), methods to generate large numbers of fertile, green transformants of two model cultivars of barley were reported (Wan and Lemaux, 1994, Plant Physiology 104:37-48). This approach involved microparticle bombardment-mediated transformation of three explants, immature embryos, young callus and microspore-derived embryos. After bombardment of these tissues, transgenic callus was selected using herbicides over several months followed by regeneration of plants. When this methodology was applied directly to commercial varieties of barley, selection of transgenic callus was straightforward. However, the callus could not be regenerated, or, if plants were regenerated, mostly albino plants were recovered. Thus, compared to model varieties these commercial lines regenerated poorly and their regenerative potential decayed rapidly over time. The low efficiencies probably resulted from the fact that only a few cells in the explant were stably transformed by bombardment and only a few cells in the explant were actually totipotent and capable of giving rise to fertile, green plants.

Optimizing Tissue Culture and Microprojectile Bombardment Procedures with Commercial Cultivars of Barley

Because of these limitations, efforts were undertaken to increase the frequency of regeneration of the cultured tissue by manipulating the timing, variety and concentration of several media components, including phytohormones, auxins and cytokinins, and certain micronutrients. Conditions were identified for maximal regenerability, which included, among other changes, the use of an intermediate step which resulted in increased shoot formation from transgenic callus. These modifications were then used utilized in transformation efforts on the commercial genotype (Cho, Jiang and Lemaux, unpublished results). Bombardment conditions were also altered to optimize for maximal callus response following bombardment; this was achieved by lowering the rupture pressure and increasing the distance between the stopping plate and the target tissue (Koprek and Lemaux, unpublished results). Selection conditions were also modified to allow more time for the tissue to recover after bombardment before imposing selection. Tissue was exposed to low light levels during the early phases of selection, reducing the numbers of albino plants dramatically (Cho, Jiang and Lemaux, unpublished results). The use of the optimized media, selection and bombardment conditions resulted in a reproducible transformation procedure for several commercially important barley cultivars.

Detrimental Effects of Tissue Culture on Barley

Plants deriving from tissue culture frequently accumulate heritable genetic changes that lead to negative field performance. In barley, analysis of the agronomic performance of tissue culture-generated wild-type and transgenic plants showed that they frequently accumulate heritable genetic changes that negatively impact their field performance. This was first documented by quantitating six agronomic attributes (heading date, height, lodging, yield, test weight and percent plump kernels) in plants from six varieties that had been cultured but were not transformed (Bregitzer and Poulson, 1995, Crop Science 35:1144-1148); all varieties studied were negatively impacted in the fifth generation by only four weeks of *in vitro* culture. A subsequent study looked at the impact on agronomic performance of plants that derived from the transformation process, which includes bombardment of target tissue (immature embryos) and subsequent culturing *in vitro* during selection (Bregitzer, Halbert, and Lemaux, manuscript submitted). The impact observed in the transgenic plants at the fourth generation was exacerbated over

that seen in plants after tissue culture alone. This negative impact of the tissue culture and transformation processes on the performance of transgenic lines will lead to a significant reduction in the utility of these technologies for agronomic improvement of barley. Although back-crossing to non-manipulated germplasm can often eliminate these problems, this requires considerable additional effort and is likely not feasible with tree species. Therefore, it is imperative to understand the cause(s) and minimize the effects of the variation generated during the *in vitro* culturing period.

Improved In Vitro Culture Systems for Transformation of Barley

Current studies are aimed at minimizing such heritable genetic variation through the use of a different target tissue for transformation. The plants that were assessed for somaclonal variation (Bregitzer and Poulson 1995; Bregitzer, Halbert and Lemaux, manuscript submitted) derived from culturing immature embryos as the source of totipotent cells; new studies have focused on using excised and cultured apical meristems as a target tissue for transformation. In collaboration with P. Bregitzer (USDA-ARS, Aberdeen ID), experiments were initiated to investigate the hypothesis that apical meristems might reduce the incidence of heritable genetic change. Plants derived from embryogenic callus and cultured shoot meristems of two varieties of barley have been analyzed for heritable changes at the molecular level. These studies show that plants regenerated from cultured meristems are nearly identical in methylation pattern to control uncultured plants, while plants derived from the standard published culture protocol (Wan and Lemaux, 1994) using immature embryos showed substantial methylation changes (Zhang, Zhang, Bregitzer and Lemaux, unpublished results). This suggests that cultured meristems might provide an improved target tissue for transformation efforts since it would result in fewer methylation changes and therefore possibly reduce the number of heritable mutational changes in regenerated barley. This material is currently being analyzed in field experiments in order to correlate the degree of molecular variability observed with agronomic performance.

Genomic and Gene Expression Instability Associated with Transformation

Introduction of exogenous DNA can have at least two drawbacks. One is that insertions occur randomly and some are detrimental for gene expression due to positional effects. This occurs with both *Agrobacterium*-mediated and direct DNA introduction methods. Another limitation with direct DNA methods is that the DNA following its introduction into the cell is often linked in multiple tandemly arrayed copies. Having multiple copies of introduced genes closely linked in the genome can lead both to gene inactivation and genetic instability, although this is not likely to be the only cause of variable gene expression.

Approaches to Limit Problems Associated with Microprojectile Bombardment

Current efforts in the laboratory are focused on the use of microprojectile bombardment with maize transposable elements as gene delivery vehicles (McElroy, Louwerse, McElroy and Lemaux, 1997, The Plant Journal, 11:157-165). This approach has several advantages. First, it should allow the generation of numerous independent insertions of introduced genes from a small number of original transformants. This is valuable because of the need to generate large numbers of events to compensate for potential detrimental positional effects. Second, this approach can lead to single copy insertions, which can lessen the possibility for genetic and gene expression instability in the final commercial product. Third, it is necessary to have selectable marker genes included with your gene of choice in order to identify transformed cells, but the elimination of potentially undesirable linked selection genes (i.e., herbicide- or antibiotic-resistance) is often desirable for commercialization purposes due to regulatory and environmental/public safety concerns. Removing the selectable marker

gene from the genome can be accomplished with the transposon approach but is not easily accomplished otherwise. Fourth, this system is also being developed as a vehicle to tag genes of agronomic and basic importance in barley.

An alternative transformation procedure utilizing *Agrobacterium tumefaciens* for transforming barley is also being tested in the laboratory. *Agrobacterium tumefaciens* is an efficient way to transfer DNA into plant cells and results in many single-copy insertions. Until recently *Agrobacterium*-mediated transformation of cereals was not possible.

SUMMARY AND RECOMMENDATIONS FOR FOREST TREES

The aforementioned areas of study are being pursued in an attempt to understand the basis for many important aspects of the transformation system and transgene and transgene expression stability. An understanding of these parameters is necessary in order for genetic engineering strategies to have optimal utility for commercial germplasm improvement. Of particular importance to the genetic engineering of forest tree species is the development of high quality tissue culture methods for transformation procedures. These tissue culture methods must work in commercially important germplasm as well as have minimal rates of heritable somatic mutation and epigenetic changes.

The rate of somatic mutation seems to be enhanced when totipotent cells redifferentiate an organized meristem or embryo. It is well known that angiosperm trees regenerated through tissue culture produce plants with significant somatic mutations and or epigenetic changes. This is also true for the spruces (Isabel et al., 1996, Am. J. Botany, 83:1121-1130). In contrast, pine trees regenerated from tissue culture have a very low rate, if any of somatic mutations (Pullman and Becwar, personal communication). One explanation for this difference might be that pine somatic embryos are normally derived from the natural polyembryony program and no redifferentiation occurs. Thus, developing tissue culture systems that minimize somatic mutation and epigenetic changes in angiosperm trees is a fundamental barrier for implementing biotechnological methods. The insights provided by the work in barley indicates that there might be a fundamental explanation for the differences in heritable somatic mutation among explants.

Transformation procedures must rapidly produce plants that stably express genes of interest with minimal effect on other important agronomic traits that affect yields. Genetic transformation of many commercially important *Populus* species is relatively inefficient. Although the tissue culture procedures with commercially important clones are being improved, another explanation for this inefficiency is the low level of transgene expression; especially selectable marker genes and inadequate selection schemes. The analysis of gene regulation in tree species is in its infancy. Much work needs to be done to achieve reliable and stable constitutive, inducible, organ- and cell type-specific expression in genetically engineered trees.

In summary, although the ability to transform given species might be available for a particular plant, many aspects of the process must be optimized in order to take full advantage of genetic engineering technologies for the production of the new valuable commercial germplasm.

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