Genetic Engineering of Reproductive Sterility in Walnut

Bei Fei and Richard Meilan

Hardwood Tree Improvement and Regeneration Center
Department of Forestry and Natural Resources and USDA-Forest Service
Pfendler Hall, 175 W. State Street, Purdue University
West Lafayette, IN 47907-2061

Abstract

In order to reduce the costs associated managing plantations, fine hardwoods are being genetically engineered for a variety of commercially important traits. However, before transgenic trees can be deployed commercially, an effective bio-confinement system is likely to be required by Federal regulators. Engineering reproductively sterility is one way of accomplishing this objective. Lengthy juvenile periods have been a serious impediment to the development of reliable sterility systems for forest trees. Having an early-flowering genotype would allow for more rapid progress in this area. We recently gained access to certain genotypes of Persian walnut (*Juglans regia* L.) that produce apical inflorescence carrying mostly hermaphroditic flowers in vitro within three months (9-18 weeks) of germination. In addition, Persian walnut is easier to transform and regenerate than black walnut (*J. nigra* L.). Thus, the early-flowering Persian walnut is being used as a model system to test constructs associated with three strategies (cell ablation, dominant negative mutations as well as RNAi) that were developed to impart reproductive sterility in various tree species. Protocols for regenerating Persian walnut has been tested and optimized. Dose-response tests have also been undertaken to determine the most effective kanamycin concentration for selecting transformants. Because the two walnut species are closely related, strategies that work in Persian walnut are also very likely to work in black walnut. Once sterile trees have been regenerated, we will evaluate the durability of this introduced trait under field conditions.

Objectives

Develop a stable and reliable system to genetically engineer reproductive sterility in early-flowering Persian walnut with *Agrobacterium*-mediated transformation, using it as a model system for genetic engineering of other walnut species.

Determine the efficiency and stability of various sterility-engineering constructs and mechanisms.

Introduction

Genetic interactions that regulate the inflorescence and floral development of plants

According to the cell proliferation and differentiation patterns, plant meristems are classified as vegetative (VM) which produces leaves and inflorescence (IM) which gives rise to the third type, floral (FM) (Shannon *et al*., 1993). When a plant grows to maturity, its VMs are converted into IMs for the reproduction. The IMs are then converted to FMs, which are competent to produce whorled floral organs. Floral homeotic genes encode products that regulate other genes important in the transition from vegetative to reproductive growth, such as directing the formation of the four flower organ whorls. Those regulatory genes such as *TERMINAL FLOWER 1* (*TFL1*), *FLOWERING LOCUS T* (*FT*), *LEAFY* (*LFY*), *AGAMOUS* (*AG*), *APETALA1* (*AP1*) and *AP2*, *AP3*, as well as *PISTILLATA* (*PI*), are potential candidates for engineering sterility (Meilan *et al*., 2001).

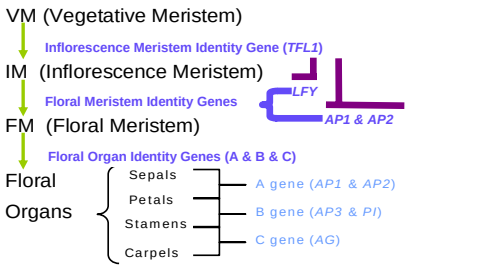
Methods used to block flower development

There are three common ways to achieve reproductive sterility:

Cell ablation. Use floral tissue-specific promoters to drive the expression of a cytotoxin gene, resulting in the death of cells that are destined to become floral tissues.

Dominant negative mutations (DNMs). They are used to inhibit the function of the wild-type floral gene product by over-producing a mutant gene product which has an inhibitory effect on the native protein (Herskowitz, 1987).

RNA interference (RNAi). Recent studies in a variety of eukaryotic organisms have shown that double stranded RNA (dsRNA) is an inducer of homology-dependent gene silencing, and use of dsRNA to induce silencing has been termed RNA interference (RNAi) (Hannon, 2002).



Model for floral homeotic gene involvement in the transition of meristems, and the formation of floral organs according to the "ABC model" (derived from Brunner *et al.*, 1998).

Materials and Methods

Somatic embryos of early-flowering phenotype of Persian walnut are being used as a starting material for *Agrobacterium*-mediated transformation. There are four embryo lines (8101-7, 8102-31, 8102-36, 8103-15), which were initiated from three Persian walnut trees, designated as 8101, 8102, and 8103, respectively. These were generated by hybridization of two early-flowering genotypes: 85-8 and 85-10. Ten binary vectors, including three DNM constructs, one RNAi construct, four *GUS* (reporter gene)-containing constructs, all driven by floral-specific promoters, will be transformed into the somatic embryos via an *Agrobacterium*-mediated transformation protocol (provided by Chuck Leslie, UC Davis). Two flowering-time vectors, containing *TFL1* and *FT* cDNA are under construction.

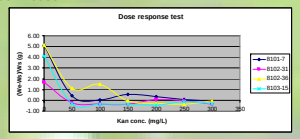
Dose-response tests have been undertaken to determine the effective kanamycin (Kan) concentration to select transformants in vitro. The Kan concentration used should effectively prevent the secondary embryogenesis from forming on the untransformed primary embryos.

The regeneration protocol of Persian walnut has also been modified. Plants can be regenerated from somatic embryos for some genotypes. In order to be more efficient in regenerating plants from our experimental lines, several different treatments are being tested.

Preliminary Results

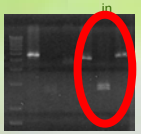
1. Regeneration

2. Dose-response results



3. Flowering-time construct assembly

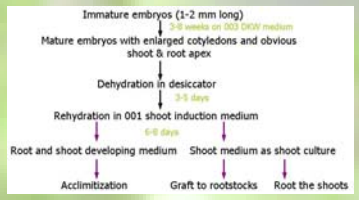
Construct (pFB-TFL1) assembly strategy



pFB001 verified by restriction digestion

Conclusions and Discussions

1. Two strategies for regeneration



2. A less stringent Kan concentration (200 mg/L) will be first used to select the transformants. After several subcultures, a more stringent conc. (250 mg/L) will be used to prevent

Literature Cited

Brunner A.M., R. Mohamed, R. Meilan, L.A. Sheppard, W.H. Rottman, S.H. Strauss. 1998. Genetic engineering of sexual sterility in shade trees. *Journal of Arboriculture* 24(5):263-273.

Herskowitz Ira. 1987. Functional inactivation of genes by dominant negative mutations. *Nature* 329:219-222.

Meilan, R., A. Brunner, J. Skinner, and S. Strauss. 2001. Modification of Flowering in Transgenic Trees. Pages 247-256 in: *Molecular Breeding of Woody Plants. Progress in Biotechnology series.* A. Komamine and N. Morohoshi, eds. Elsevier Science BV, Amsterdam.

Shannon, S. and D.M. Wagner. 1993. Genetic interactions that regulate inflorescence development in *Arabidopsis*. *The Plant Cell* 5:639-655.