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BLIGHT RESISTANCE TECHNOLOGY: TRANSGENIC APPROACHES

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Abstract: The technology needed to produce a blight-resistant, transgenic American chestnut tree has come to fruition. Many technical hurtles have been overcome and the first transgenic American chestnut trees are expected to be in the greenhouse within one to two years. These first trees will contain an oxalate oxidase transgene from wheat, under control of a regulated promoter from soybean. This will be the first of several putative resistance-enhancing transgene constructs to be tested for its ability to confer chestnut blight resistance. During the field trial phase of this research, we hope to include a few educational research plots accessible to the public under controlled conditions. In this way we can enhance the transparency of this project by allowing the general public to see first hand the results of this research. Once the transgenic trees are approved for release, they will enter a restoration program to rescue as much of the remaining genetic diversity in the surviving American chestnuts. This research should also help pave the way for the restoration of other threatened tree species through the use of biotechnology.

INTRODUCTION

Due to the movement of plant materials around the world, many exotic diseases and pests have threatened North American forests over the past century. Tree diseases such as white pine blister rust, chestnut blight, beech scale complex, Dutch-elm disease, butternut canker, dogwood anthracnose, just to name a few examples, have caused significant losses of trees in our forests and in urban settings. Although the United States has established several regulatory safeguards, diseases, such as the newly discovered sudden oak death, sometimes get through. It is likely that new introductions of diseases and pests will continue into the future. In an effort to restore species from past and ongoing epidemics, researchers are beginning to apply biotechnological techniques to forest species. In our labs, we are taking a transgenic approach to enhance pathogen resistance in American elm and in American chestnut. This report will describe the progress made to date and discuss the possible future of blight-resistant, transgenic American chestnut trees.

TRANSGENE DESIGN

When choosing transgenes to enhance pathogen resistance, and to play a vital role in the restoration of a valuable tree species such as the American chestnut (*Castanea dentata* (Marsh.) Borkh.), several considerations need to be taken into account. First, if the tree produces an eatable product, like the nut of the American chestnut, it must be equally as safe to eat for humans and wildlife as the nuts from non-transgenic trees. Second, the transgenic tree should have effective and durable resistance to the blight. Third, the transgenic tree should retain all the positive traits of the American chestnut so that it can be reestablished in its natural niche in the environment. Fourth, the transgenic tree should be amenable to a restoration program. For example, it should be able to recapture a significant portion of the remaining genetic diversity in the surviving population. Lastly, it needs to be acceptable to the general public, i.e. the majority of the public should view the trees positively. In our transgene designs, we have considered all these aspects when choosing what to use.

Several genetic components needed to construct a variety of putative resistance enhancing genes are currently available (Powell et al. 1995; Powell and Maynard 1997; Powell et al. 2000; Liang et al. 2001; Connors et al. 2002; Connors et al. 2002; Liang et al. 2002). More putative resistance enhancing genes are being reported each year and someday the resistance genes from the Asian chestnuts trees might be cloned, enhanced, and used to transform American chestnut trees. But to save time and space, this report will focus only on the transgene construct that will be used first to produce a transgenic American chestnut. If this construct fulfills all the necessary criteria for producing a blight-resistant American chestnut, then this might be the only construct needed, but if this transgenic construct is not as effective as needed, many other genes and gene promoters are available. The first transgenic American chestnuts will contain a three-gene cassette as show in the binary vector pVSPB-OxO (fig. 1).

This construct contains an oxalate oxidase (OxO) encoding gene from wheat (Lane et al. 1986; Lane 1994). This gene was selected because it comes from a familiar plant that is consumed by most Americans every day and therefore brings with it a sense of public acceptability. It is also being researched for use to enhance pathogen resistance in other transgenic crop species, which should help with the regulatory approval as government reviewers become familiar with this transgene's properties. Lastly, its mechanism for enhancing resistance appears to be well suited for producing effective and durable resistance to the chestnut blight.

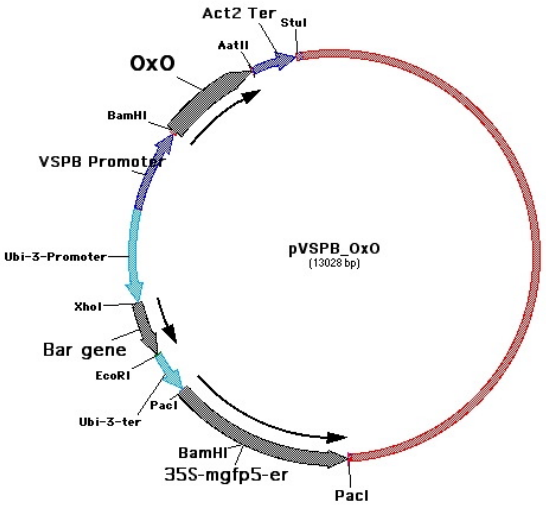

Figure 1. Plasmid map of the binary vector pVSPB-OxO.

Oxalate oxidase catalyzes the degradation of oxalic acid into H_2O_2 and CO_2. Similar enzymes have been found in several plant species such as barley and wheat and are expressed during germination, stress, and fungal infection (Dumas et al. 1995; Zhang et al. 1995; Hurkman and Tanaka 1996; Hurkman and Tanaka 1996). This enzyme is of interest because *Cryphonectria parasitica*, the chestnut blight fungus, produces large amounts of oxalate at the canker margin, which helps lower the pH to toxic levels and binds calcium (Roane et al. 1986). Oxalic acid, or oxalate, has also been associated with pathogenesis in other fungi (Noyes and Hancock 1981; Marciano et al. 1983; Cessna et al. 2000). Therefore, it is reasonable to hypothesize that neutralizing oxalic acid will enhance resistance to these fungi. One byproduct of the oxalate degradation, H_2O_2, could induce a separate defense mechanism, which would only be activated when the fungus produces oxalate. H_2O_2 has been shown to be a signal molecule that induces a plant's natural defense system (Lane et al. 1993; Lane 1994) and could enhance resistance in transgenic plants (Wu et al. 1995). Previously, we had cloned the wheat oxalate oxidase transgene into a model tree species, hybrid poplar, and shown that it could enhance resistance to another oxalate producing pathogen, *Septoria musiva* (Liang et al. 2001). Other researchers have cloned this same gene into soybean and shown enhanced resistance to the white mold fungus, *Sclerotinia sclerotiorum* (Cober et al. 2003). Recently, transgenic callus from American chestnut expressing the oxalate oxidase transgene and grown in the presence of oxalic acid, was shown to retain its ability to produce lignin at normal levels. Oxalic acid would significantly inhibit lignin formation in the non-transformed controls (Welch, Stipanovic, Maynard, and Powell, unpublished). Lignin synthesis is necessary to compartmentalize fungal infections in plants. Therefore, since the wheat oxalate oxidase gene will have multiple resistance enhancing effects, we believe it will likely enhance blight resistance in American chestnut and be sustainable.

Attached to the oxalate oxidase transgene is a regulated promoter that can control which tissues in the plant can express the gene. In this construct, the promoter from the soybean vegetative storage protein B (VSPB) gene was chosen because its expression is induced by sucrose and by wounding and repressed by auxins (Mason et al. 1993; DeWald et al. 1994). Its expression pattern therefore is primarily in the stems and wound sites, the tissues that can be infected by the chestnut blight, and it is not expected to be produced in significant amounts in the nuts. The expression of the oxalate oxidase transgene in pVSPB-OxO has been tested in *Arabidopsis* and shows vascular expression as expected (fig. 2).

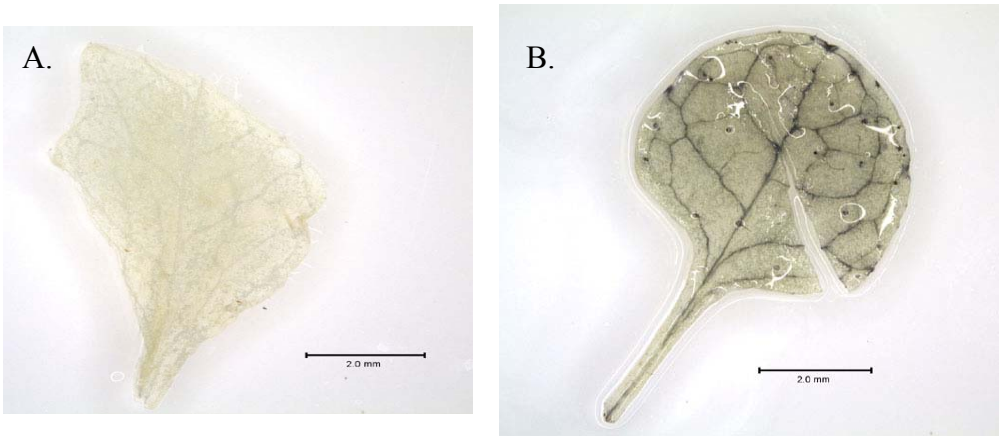

Figure 2. Oxalate oxidase assays of non-transformed Arabidopsis (A) and Arabidopsis transformed with pVSPB-OxO (B). The dark coloration in the vascular tissues seen in B is a positive result.

In addition to the putative resistance-enhancing gene, there are two other transgenes commonly found in binary vectors. The first is the BAR gene (Figueira Filho et al. 1994; Metz et al. 1998), which confers specific resistance to phosphinothricin (PPT), the active ingredient in the herbicide Finale®. In plant transformations, this gene is used for selection of transformed cells. In an American chestnut restoration project, this gene will also have a second useful function. To save as much of the genetic diversity, including rare alleles, in the surviving population of American chestnuts, a restoration program would need to out-cross to as many of these trees as possible. To do this efficiently, the transgenes must convey resistance in the hemizygous state, meaning full resistance from a single copy. If a hemizygous tree were to be out-crossed to a non-transgenic tree, only half of the resulting seeds would contain the transgenic resistance cassette. This is where the herbicide resistance would be useful. Nurserymen could plant all the seeds and as they sprout, spray them with Finale®. Only the transgenic trees would survive. This would save a lot of labor and money, and would increase the efficiency of a restoration program. This would also allow easy identification of the transgenic trees in the field by a technique called spotting. In this technique, a small drop of Finale® is spotted on a freshly excised leaf. If the plant is transgenic, no necrotic spot will appear, but after a few days, the non-transgenic plants will display a necrotic spot on the leaf.

Lastly, in pVSPB-OxO there is a gene encoding a green fluorescent protein (Haseloff et al. 1997). When illuminated by ultraviolet or blue light, this protein will fluoresce green and can be detected using specific filters. This gene has greatly enhanced our ability to optimize the transformation procedure because it allows visual identification of the transformed tissues without damaging them. This gene is currently being used to optimize our transformation procedures but may or may not be in the final transgenic tree released. Although this gene is harmless, we will gage public acceptance before using it in the released trees.

CHESTNUT SOMATIC EMBRYO TRANSFORMATIONS

This year, 2004, marks one hundred years since the discovery of the chestnut blight (Merkel 1906). This is also the year in which a method has been developed that can consistently produce transgenic American chestnut. Over the past fourteen years, foundation research has been accomplished in Dr. Maynard's and Dr. Merkle's labs (Merkle 1991; Carraway et al. 1997; Xing et al. 1997; Xing et al. 1999). This year, two advances have greatly improved the transformation protocol. The first is the use of GFP to identify and follow transformed tissues. The second was to add a desiccation step to the transformation procedure (Cheng 2003). To date, nine lab members and students have been able to transform chestnut embryos. Some of the best looking transgenic embryos are beginning to develop (fig. 3). Currently, the first transgenic lines of embryos are being propagated and a portion will be stored cryogenically in Dr. Merkle's lab (Holliday 2000). The remainder will begin the process of regeneration into whole plants.

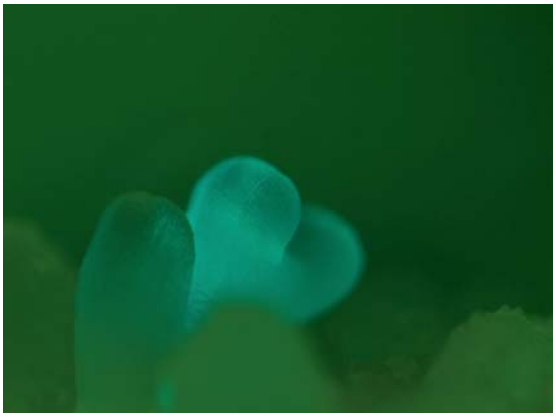

Figure 3. Example of an American chestnut somatic embryo transformed with pVSPB-OxO expressing GFP (transformed & photographed by Ron Rothrock, Dr. Maynard's graduate student).

NEXT STEPS

In parallel with the transformation work, we have been conducting studies on propagating American chestnut somatic-embryo-derived and apical-meristem-derived plantlets and acclimatizing those plantlets for establishment in the field (LaPierre 2003). The first non-transgenic, micropropagated American chestnut plantlets were established in a bare-root nursery in 1997 and were lifted, examined for root morphology (fig. 4), and transplanted to the field in 2001.

The next step in the process of evaluating the new transgenic somatic embryos will be to first multiply and germinate individual embryos (Merkle et al. 1991, Carraway et al. 1997; Xing et al. 1999), or if germination is low, micropropagate them (Xing et al. 1997). Once a sufficient supply of transgenic plantlets has been produced, they will be acclimatized and grown in the greenhouse (Bickel *et al* 2000) until they have a minimum stem caliper of 3 mm and then screened for transgene expression in the stem tissues. The trees that express the transgene as expected will then be tested for blight resistance. Those transformation events producing trees with a high level of blight resistance will be planted in field trials and evaluated against non-transgenic American and Chinese chestnut (*Castanea mollissima*) seedlings for resistance, growth characteristics, and mycorrhizal interactions.

Figure 4. American chestnut tissue-culture-derived trees (left of meter stick) and seedling controls (right of meter stick) after four growing seasons.

CONCLUSIONS

If all goes according to our projected timeline, we expect to have the first potted transgenic American chestnut trees in the greenhouse by the spring of 2005. At this time we will start the regulatory process for approved release, beginning with USDA notification of the field trials. These plants will then be hardened off and should be ready for field planting in the fall of 2005. We hope that some of the field trials can be set up as public educational demonstrations. These plantings will be in controlled areas, but will be accessible to the public so that they can observe the ongoing experiments and learn about the chestnut blight and about possible uses of forest biotechnology. The final transgene make-up of the transgenic American chestnut that will be released to the public will depend on the results from the resistance assays, field trials, regulatory approval, and public acceptance. Once approved for release, we believe that transgenic American chestnuts trees will play a key roll in the restoration of this threatened species.

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