The Commercial Potential for Genetic Engineering in Alfalfa

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Plant genetic engineering techniques, developed in the last 25 years, have been applied to the improvement of several crop species. Transgenic soybeans, corn, cotton, and canola with insect resistance and/or herbicide tolerance have been well received by U.S. farmers. Agrobacterium-mediated alfalfa transformation was first accomplished in the mid 1980's (Deak et al., 1986, Shahin et al., 1986). Since that time very efficient transformation of elite alfalfa lines has become a routine process in many labs. Alfalfa genetic engineering has proven to be a useful research tool and offers the potential to be a powerful tool for alfalfa improvement.

This paper presents a summary of the commercial potential for genetic engineering in alfalfa, the key steps required for commercialization of transgenic traits, and some of the current challenges in commercialization of transgenic alfalfa varieties.

Potential Commercial Target Traits for Alfalfa Genetic Engineering

Input traits designed to improve agronomic performance and output traits with the potential to increase forage quality or produce novel industrial/pharmaceutical proteins are the focus of current research in alfalfa genetic engineering.

The first generation biotech traits for plants use genetic engineering to incorporate transgenes that code for proteins that are directly responsible for a value-added trait. The bacterial *cp4-epsps* gene conferring glyphosate tolerance and bacterial Bacillus thuringiensis (Bt) genes conferring insect resistance are examples of such "first generation" traits. Roundup Ready alfalfa plants containing the *cp4-epsps* transgene were produced in 1998. Commercial development of RR alfalfa is underway. Coleopteran-active Bt transgenes have been incorporated into alfalfa in attempts to generate alfalfa plants resistant to the alfalfa weevil and/or clover root curculio. Transgenic alfalfa plants that contains fungal chitinase genes are being tested for resistance to a broad range of fungal pathogens. Novel enzymes have also been introduced into several crops in an attempt to increase tolerance to abiotic stresses. Transgenic alfalfa plants containing a superoxide dismutase (SOD) gene have shown increased winter survival and increased forage yield in preliminary field tests (McKersie et al., 2000). Transgenes offering potential salt tolerance have also been reported (Winicov, 2000). Austin-Phillips and Ziegelhoffer (2001) have shown that transgenic alfalfa containing a fungal phytase gene produce significant quantities of this feed enzyme and the Canadian company Medicago Inc., is looking at alfalfa as a potential factory for production of high value transgenic proteins (Khoudi et al., 1999).

A second generation of biotech traits is being explored that uses genetic engineering to alter metabolic pathways. This approach has been used in several model systems, including alfalfa. Tesfaye, et. al. 2001, report that transgenic alfalfa containing malate dehydrogenase (MDH) show increased tolerance to acid soils and high soil Al content. The Nobel Foundation Plant Biology Group has used alfalfa as a model system with transgenic knockouts as a tool to better understand lignin biosysnthesis. Guo et al., (2001a, b) report that transgenic alfalfa plants with down- regulated comethyl transferasecaffeic acid 3-O-methyltransferase (COMT) and caffeoyl CoA 3-O-methyltransferase (CCOMT) show altered lignin content and composition and improved fiber digestibility. Several groups are now working on various strategies to alter tannin biosynthesis in alfalfa to produce transgenic alfalfa plants expressing tannins in leaves and stems. Based on evidence from tannin producing forage legumes such as birdsfoot trefoil and sanfoin, a low concentration of leaf tannins can significantly slow post-harvest and ruminal protein degredation, potentially improving the efficiency of alfalfa protein utilization containing forages also have much lower potential for ruminant bloat when grazed (Albrect and Muck, 1991)

Steps in Commercialization

Once a target gene is identified, there are several steps involved in the evaluation and potential commercialization of a transgenic trait. The critical steps are outlined below:

$ *Vector construction* – the selection of the appropriate promoter, transit peptide, leader sequence, terminator, etc. will greatly effect the expression level of the target gene, and dictate the required intellectual property (IP) licenses for commercialization.

- $ *Transformation* – alfalfa has been successfully transformed with both Agrobacterium vectors and with particle bombardment. In most cases Agrobacterium-mediated transformation yields a higher frequency of the desired simple, single copy insertions. Transformation of an elite alfalfa genotype will facilitate T_0 evaluation and expedite the product development process.
- $ *Confirmation and characterization* – a construct specific PCR-based test confirms that the transformed plants contain the transgene. This is usually followed by demonstration of sexual stability of the transgene, progeny analysis as an indicator of copy number, and comprehensive Southern blot analysis for molecular characterization of the insert.
- $ *Proof of concept* – a multi-stage process that usually begins with an analysis of the initial transformant (T_0) plants in the greenhouse or growth chamber, but is usually followed by multiple location field trials to confirm the desired phenotype. This and all other field tests described in this section are regulated by USDA/APHIS and require special handling and isolation to prevent escape of the regulated transgenes.
- $ *Event selection* – evaluation of several dozen events for the desired transgenic phenotype, substantial equivalence for agronomic and forage quality traits, and "clean" molecular inserts. A small fraction of the original T_0 plants are likely to meet these combined criteria. One or more "commercial events" will be identified and submitted for regulatory approval.
- $ *Trait integration* – backcrossing the "commercial event(s)" of the transgenic trait into elite commercial lines that include the agronomic characteristics that are required for adaptation to key commercial target markets. Transgenes behave as single gene dominant traits.
- $ *Product development* – an extension of the trait integration program that includes field evaluation of transgenic populations, selection of parent clones, and the agronomic evaluation of transgenic experimental varieties typical prior to commercial variety release. The autotetraploid nature of alfalfa presents unique challenges in transgenic breeding. A multihomogenic breeding strategy has been developed for use when high trait purity (% of plants in the variety with the transgenic phenotype) is desired (McCaslin, et. al., 2002, Samac and Temple, 2002). This program uses event-specific PCR as a molecular marker to identify plants containing multiple copies of the transgene at separate independent loci.
- $ *Regulatory approval* – before U.S. commercial release, all transgenic events require approval by the USDA to confirm environmental safety and the FDA to confirm food and feed safety. The required tests include: thorough molecular characterization of the transgenic insert and the flanking genomic DNA; ecological evaluation of the transgenic trait on non-target plant populations (i.e. wild relatives or feral plants) and, data that supports the substantial equivalence of the transgenic plant compared with non-transgenic plants for key traits of the species. The collection of data, preparation of the regulatory submissions, and submission review by the agencies is a three to five year process. Regulatory approval will also likely be sought in other countries where the trait has significant economic value or where U.S. alfalfa hay products are exported.
- $ *Commercial release* – after regulatory approval commercial seed of transgenic varieties can be marketed.

<u>Challenges</u>

Although genetic engineering offers many opportunities for alfalfa improvement, there are several challenges, both technical and commercial.

- $ Transformation technology, promoters, transit peptides, selectable markers, etc. are all protected with one or more U.S. and international patents. Negotiating freedom to operate (FTO) for this package of required enabling technology is a significant challenge. In addition to the necessary enabling technology licenses, a license for the particular trait gene is required.
- $ There are very few fully characterized promoters that give high gene expression in alfalfa. The 35S CaMV promoter, widely used as a constitutive promoter in the plant biotech research community, is only marginally effective in alfalfa. A tool box of constitutive, tissue and developmentally specific promoters that are effective in alfalfa will be needed to optimize expression of various transgenes. There is currently

little research work being done in this area.

$ Alfalfa is an autotetraploid, perennial forage crop. The transgenic trait integration and trait stacking strategies developed for corn, soybeans and cotton are not appropriate for alfalfa. As discussed earlier, new breeding methods are being developed that address the unique challenges of alfalfa.

$ The successful management of the regulatory approval process requires expertise not typical in most alfalfa breeding organizations. Regulatory approval for transgenic traits in alfalfa will likely also be necessary in key countries that represent important export markets for U.S alfalfa products (e.g. Japan).

$ Public acceptance issues for transgenic crops continue to evolve rapidly and currently limit the commercial potential of the technology in some markets (e.g. Europe).

$ The commercialization process is expensive, and will limit genetic engineering in alfalfa to those traits and markets that have the most value-added potential.

Summary

Ongoing transgenic research projects offer the potential to increase the efficiency of alfalfa production and significantly increase the value of alfalfa forage. Although the process of commercialization of transgenic varieties will present both technical and commercial challenges, current and future efforts on selected value-added traits will offer the potential to significantly improve the commercial potential of the crop.

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