ALFALFA IN THE 21ST CENTURY: POSSIBILITIES AND LIMITATIONS OF GENETIC ENGINEERING

Mary K. Sledge
Forage Legume Breeder
The Noble Foundation, 2510 Sam Noble Parkway, Ardmore, OK 73401
Email: mksledge@noble.org

ABSTRACT

Research in the genetic engineering of alfalfa is currently underway to improve alfalfa for traits such as tolerance to abiotic and biotic stresses, forage quality, and herbicide resistance. Alfalfa is also being engineered to produce novel compounds for industrial and diagnostic purposes. This research will likely lead to the production of improved cultivars, as well as new uses for alfalfa.

INTRODUCTION

The term "genetic engineering" commonly refers to the artificial insertion of a gene, or portion of a gene, into an organism. Genetically engineered plants are called "transgenic" plants, indicating that a gene has been transferred into the plant. The transferred gene is referred to as a "transgene". Genetic engineering technologies are used when a desired trait, conditioned by one or more genes, isn't available in the plant species of interest, nor in a related species with which the species of interest can by hybridized. The gene can come from another plant species, or from a non-plant species, such as a bacterium. In alfalfa, the most commonly used method of gene transfer is *Agrobacterium tumefaciens* mediated gene transfer. *A. tumefaciens* is a naturally occurring soil bacterium that causes grown gall disease in many ornamental and fruit plants. The bacterium enters the plant through a wound site, and inserts a portion of its own DNA into the plant. Infected plant cells form a tumor, or gall, and use the inserted DNA to synthesize metabolites called opines, which the bacterium uses as a food source. Scientists have harnessed this natural process by removing the tumor inducing genes, and replacing them with transgenes (de la Riva et al., 1998).

TRANSGENIC RESEARCH IN ALFALFA

Abiotic Stress tolerance. Injury from environmental stresses, such as drought, freezing, flooding, and diseases is associated with the increased production of oxygen free radicals. A class of metalloproteins called superoxide dismutases (SODs) has the ability to detoxify oxygen free radicals, converting them to hydrogen peroxide and molecular oxygen. Alfalfa engineered to overexpress SOD has exhibited improved winter survival (McKersie et al., 1999), and reduced secondary injury symptoms with enhanced winter stress recovery (McKersie et al., 2000). Also under investigation is the role that sucrose could play as a cryoprotectant in increasing the winterhardiness of alfalfa. The sucrose-phosphate synthase (SPS) gene channels carbohydrate away from starch production and into sucrose accumulation. Alfalfa has been engineered to constitutively express this gene, and field-testing will determine if raising levels of sucrose will improve freezing tolerance (Shearer et al., 2002).

Soil salinity is an increasing problem on irrigated agricultural lands, particularly in hot regions of the world where the evaporation rates are high. Salts introduced by irrigation water accumulate at or near the soil surface, impairing the plants ability to take up water, and resulting in reduced plant productivity. Genes associated with salt tolerance in alfalfa have been identified. *Alfin I*, a putative transcription factor, enhances expression of the salt-inducible *MsPRP2* gene in alfalfa roots (Wincov and Bastola, 1999). Transgenic alfalfa overexpressing *Alfin I* exhibit increased plant growth and salt tolerance (Winicov, 2000).

Alfalfa is sensitive to aluminum (Al) toxicity, which results in stunted root growth and low yield. Production of organic acids is associated with Al tolerance in a wide range of plants (de la Fuente et al., 1997; Hocking, 2001). Alfalfa transformed with the citrate synthase gene, for the production of citric acid, has exhibited better growth and longer roots than nontransgenic control plants (Rosellini et al., 2002). Alfalfa transformed with the malate dehydrogenase gene exhibited increased production of citrate, oxalate, malate, succinate, and acetate, with a corresponding increase in Al tolerance (Tesfaye et al., 2001).

***Biotic Stress Tolerance*.** Plant secondary metabolites called phytoalexins have been associated with antifungal activity in alfalfa. The nature of this activity tends to be broad-spectrum (Dixon 2001), and overexpression of phytoalexin genes in alfalfa could provide defense against pathogens for which no specific source of resistance is available. Alfalfa transformed with the phytoalexin resveratrol from peanut has increased resistance to *Phoma medicaginis* (Hipskind and Paiva, 2000). Likewise, alfalfa transformants overexpressing isoflavone O-methyltransferase (IOMT) also show increased resistance to *P. medicaginis* (He and Dixon, 2000). Reduced severity of infection by *Phytophthora megasperma* was achieved in alfalfa overexpressing an inducible beta-1, 3- glucanase, a pathogenesis-related (PR) protein (Masoud et al., 1996).

Transgene mediated insect resistance has been reported in alfalfa with the insertion of an insect proteinase inhibitor. Transformants expressing the anti-elastase protease inhibitor (PI) from *Maduca sexta* exhibited reduced onset of thrip predation (Thomas et al., 1994). *Bt* genes code for crystalline (*Cry*) proteins, which breakdown in the gut of lepidopteran insects, and cause paralysis of the digestive system, and death of the insect (Gill et al., 1992). *Bt* transgenes have been transferred to alfalfa for the potential control of alfalfa weevil and clover root curculio (McCaslin, 2002).

Herbicide resistance. The 5-enolpyruvylshikimate –3-phosphate synthetase (EPSPS) gene is necessary for the production of amino acids essential for plant growth. Glyphosate herbicides, such as Roundup ^{TM}, work by inhibiting this enzyme. A form of this enzyme that is not sensitive to glyphosate can be found in the bacterium *E. coli*. Transferring this form of the enzyme to plants results in resistance to glyphosate herbicides (Padgette et al., 1995). Currently, alfalfa containing these transgenes is not commercially available. However, commercial development of glyphosate resistant alfalfa is currently underway (McCaslin, 2002).

***Forage Quality*.** Low levels of sulfur-rich amino acids are a major limitation to wool growth in sheep. In order to improve wool production, alfalfa has been transformed with genes coding for

proteins rich in sulfur-containing amino acids (Higgins et al., 1989). A ruminally stable, sunflower seed albumin gene has increased the sulfur amino acid content of alfalfa, and adding a endoplasmic reticulum retention signal to the transgene greatly increased the accumulation of the sunflower seed albumin in alfalfa leaves (Tabe, 1995). Maize gamma zein, which codes for a sulfur amino acid-rich seed storage protein, has also been introduced into alfalfa in order to improve its nutritional quality for wool production (Bellucci et al., 1997; Bellucci et al., 2001).

Lignin is the major structural component of plant secondary cell walls. It lends strength to stems, but is poorly digestible. Increasing the digestibility of lignin could improve the nutritive quality of forages. Several enzymes in the phenylpropanoid pathway leading to the biosynthesis of lignin have been manipulated to decrease lignin quantity or alter lignin composition, both of which affect alfalfa digestibility. Cinnamyl –alcohol dehydrogenase, which catalyzes the final step in lignin biosynthesis, was downregulated with an antisense construct. The quantity of lignin was not changed, however the composition of the lignin was altered (Baucher et al., 1999). Down regulation of either caffeic acid 3-O-methyltransferase (COMT) or caffeoyl coenzyme A 3-O-methyltransferase (CCOMT) led to decreased lignin content and altered lignin composition. The altered lignin of each was improved for digestibility, however, the downregulation of CCOMT led to a greater improvement in digestibility (Guo et al., 2001a; Guo et al., 2001b).

Condensed tannins are secondary metabolites that complex with protein, reducing stable foaming in the rumen that occurs when proteins are rapidly digested, and thus reducing the incidence of bloat in ruminant animals (Li et al., 1996). Adding condensed tannins to alfalfa forage reduces protein solubility (Julier et al., 2002). Genes for condensed tannins have been isolated from *Medicago truncatula*, an annual medic closely related to alfalfa, and *Arabidopsis thaliana* (Xie et al., 2002). Isolation of these genes is the critical first step towards the production of alfalfa genetically engineered for bloat safety.

Production of Novel Compounds. Alfalfa has been engineered to produce a variety of novel proteins for industrial use. Use of alfalfa for the production of industrial enzymes has the advantage of producing large quantities of enzyme at a relatively low cost (Austin and Bingham, 1997). Alfalfa produced phytase is able to replace inorganic phosphorus in poultry and swine feed, and reduce the levels of phosphate in the manure (Koegel et al., 1999). Alfalfa produced cellulases could significantly reduce the cost of converting biomass to alcohol (Ziegelhoffer et al., 1999). Alfalfa has also been engineered to produce monoclonal antibodies for diagnostic use. The anti-human IgG antibody, a commonly used reagent in blood banks, was produced in alfalfa and found to be functionally identical to the hybridoma produced antibody. Antibody production was stable over repeated harvests and in drying hay. (Khoudi et al., 1999). A vaccine for transmissible gastroenteritis (TGE) in swine has been produced in alfalfa, and could lead to an inexpensive, edible vaccine (Tuboly et al., 2000).

LIMITATIONS OF GENETIC ENGINEERING

The rate of gene discovery and characterization is the most limiting aspect of genetic engineering. There are several approaches to the gene discovery process (Miflin 2000). A genomics based approach involves a search for homology with known genes from other species and/or an analysis of gene expression under differing environmental conditions. Genes for

specific traits may also be tagged by associating DNA markers with trait expression. Once genes are identified, they must be isolated, or cloned, and then introduced into a plant species, and their effect on the trait expression in the plant analyzed. Alternatively, known genes from metabolic pathways can be isolated, and either suppressed or overexressed, and the effect on plant function analyzed. Years of field-testing must be carried out as for any commercial cultivar, but must be done in compliance with governmental regulations so as to prevent movement of transgenes into weedy relatives. Complicating commercialization of a genetically engineered crop are the intellectual property rights associated with the many of the tools of genetic engineering, such as plant promoters and selectable markers. The cost of licensing these tools can be prohibitive, making genetic engineering currently feasible only for very high value traits. There is currently some lack of public acceptance of genetically engineered crops for human consumption. Since alfalfa is primarily consumed by livestock, this may not be a limiting factor for production of genetically engineered alfalfa.

References

Austin, S., and E.T. Bingham. 1997. The potential use of transgenic alfalfa as a bioreactor for the production of industrial enzymes. p. 409-424. *In* B.D. McKersie and D.C.W Brown (eds). Biotechnology and the Improvement of Forage Legumes. CAB International, Wallingford, UK.

Baucher, M., M. A. Berard-Vailhe, B. Chabbert, J.M. Besle, C. Opsomer, M. van Montagu, and J. Botterman. 1999. Down-regulation of cinnamyl alcohol dehydrogenase in transgenic alfalfa (Medicago sativa L.) and the effect on lignin composition and digestibility. Plant Mol. Biol. 39:437-447.

Bellucci, M., B. Lazzari, A. Viotti, and S. Arcioni. 1997. Differential expression of a gamma-zein gene in Medicago sativa, Lotus corniculatus, and Nicotiana tabacum. Plant Sci. Limerick. 127:161-169.

Bellucci, M., F. de Marchis, and S. Arcioni. 2001. Expression of maize genes in transgenic forage legumes. p. 85-87. *In* I. Delgado and J. Lloveras (eds). Quality in Lucerne and Medics for Animal Production. Proceedings of the XIV Eucarpia Medicago spp. Group Meeting, Zaragoza and Lleida, Spain, 12-15 September 2001. Options Mediterraneennes. Serie A, Seminaires Mediterraneens.

de la Riva, G.A., J. Gonzales-Cabrera, R. Vazquez-Padron, and C. Ayra-Pardo. 1998. The *Agrobacterium tumefaciens* gene transfer to plant cell [Online]. EJB Electronic Journal of Biotechnology. Available at http://www.ejb.org/content/vol1/issue3/full/1/bip/ (verified 13 November 2002).

Dixon, R.A. 2001. Natural products and plant disease resistance. Nature 411:843-847.

Gill, S.S., E.A. Cowles, and F.V. Pietrantonio. 1992. The mode of action of *Bacillus thuringiensis* endotoxins. Ann. Rev. Ent. 37:615-636.

Guo, D., F. Chen, J. Wheeler, J. Winder, S. Selman, M. Peterson, and R.A. Dixon. 2001b. Improvement of in-rumen digestibility of alfalfa forage by genetic manipulation of lignin O-methyltransferase. Trans. Res. 10:457-464.

Guo, D.J., F. Chen,, K. Inoue, J.W. Blount, and R.A. Dixon. 2001a. Downregulation of caffeic acid 3-O-methyltransferase and caffeoyl CoA 3-O-methyltransferase in transgenic alfalfa: impacts on lignin structure and implications for the biosynthisis of G and S lignin. Plant Cell. 13:73-88.

He, X.Z., and R.A. Dixon. 2000. Genetic manipulation of isoflavone 7-O-methyltransferase enhances biosynthesis of 4'-O-methlated isoflavonoid phytoalexins and disease resistance in alfalfa. Plant Cell. 12:1689-1702.

Higgins, T.J., P.A. O'Brien, D. Spencer, H.E. Schroeder, H. Dove, and M. Freer. 1989. Potential of transgenic plants for improved amino acid supply for wool growth. p. 441-445. *In* G. E. Rogers and P.J. Reis (eds). The Biology of Wool and Hair. Chapman and Hall, London, UK.

Hipskind, J.D., and N.L. Paiva. 2000. Constitutive accumulation of a resveratrol-glucoside in transgenic alfalfa increases resistance to *Phoma medicaginis*. Mol. Plant-Microbe Interact. 13:551-562.

Hocking, P.J. 2001. Organic acids exuded from roots in phosphorus uptake and aluminum tolerance of plants in acid soils. Adv. Agron. 74:63-97.

Julier, B., M. Lila, C. Huyghe, P. Morris, G. Allison, and M. Robbins. Effect of condensed tannins on protein solubility in legume forages. The 38th Report of the North American Alfalfa Improvement Conference, Sacramento, CA. 27-31 July. 2002. (Available on-line at http://www.naaic.org/Meetings/National/2002meeting/2002Abstracts/JulierCT.pdf.) (verified 7 Nov. 2002.)

Khoudi, H., S. Laberge, J.M Ferullo, R. Bazin, A. Darveau, Y Castonguay, G. Allard, R. Lemieux, and L.P. Vezina. 1999. Production of a diagnostic monoclonal antibody in perennial alfalfa plants. Biotech. and Bioeng. 64:135-143.

Koegel, R.G., R.J. Straub, S. Austin-Phillips, M.E. Cook, and T.D. Crenshaw. 1999. Alfalfa-produced phytase for supplementation of poultry and swine rations. ASAE-CSAE-SCGR Annual International Meeting. 18-21 July 1999. Toronto, Ontario, Canada.

Li, Y., G.Tanner, and P. Larkin. 1996. The DMACA-HCl protocol and the threshold proanthocyanidin content for bloat safety in forage legumes. J Sci. Food Agric. 70:89-91.

Masoud, S.A., Q. Zhu, C. Lamb, and R.A. Dixon. 1996. Constitutive expression of an inducible beta-1, 3-glucanase in alfalfa reduces the disease severity caused by the oomycete pathogen *Phytophthora megasperma* f. sp. *medicaginis*, but does not reduce disease severity of chitin-containing fungi. Trans. Res. 5:313-323.

McCaslin, M. 2002. The commercial potential for genetic engineering in alfalfa. The 38th Report of the North American Alfalfa Improvement Conference, Sacramento, CA. 27-31 July. 2002. (Available on-line at http://www.naaic.org/Meetings/National/2002meeting/2002Abstracts/McCaslinSymposium.pdf.) (verified 7 Nov. 2002.)

McKersie, B.D., S.R. Bowley, and K.S. Jones. Winter survival of transgenic alfalfa overexpressing superoxide dismutase. Plant Phys. 119:839-847.

McKersie, B.D., J. Murnaghan, K.S. Jones, S.R. Bowley. 2000. Iron-superoxide dismutase expression in transgenic alfalfa increases winter survival without a detectable increase in photosynthetic oxidative stress tolerance. Plant Phys. 122:1427-1437.

Miflin, B. 2000. Crop improvement in the 21st century. J. Exp. Bot. 51(342):1-8.

Padgette, S.R., K.H. Kolacz, X. Delannayh, D.B. Re, B.J. LaValle, C.N. Tinius, W.K. Rhodes, Y.I. Otero, G.F. Barry, and D.A. Eichholtz. 1995. Development, identification, and characterization of a glyphosate-tolerant soybean line. Crop Sci. 35:1451-1461.

Rosellini, D., P. Barone, J. Bouton, P. LaFayette, M. Sledge, F. Veronesi, and W. Parrot. 2002. Aluminum tolerance in alfalfa with the citrate synthase gene. The 38th Report of the North American Alfalfa Improvement Conference, Sacramento, CA. 27-31 July. 2002. (Available on-line at http://www.naaic.org/Meetings/National/2002meeting/2002Abstracts/Rosellini.pdf.) (verified 7 Nov. 2002.)

Shearer, H.L., J. Friedberg, and S. R. Bowley. 2002. The effect of enhanced sucrose-phosphate synthase (SPS) activity on the low-temperature survival of alfalfa (*Medicago sativa* L.). The 38th Report of the North American Alfalfa Improvement Conference, Sacramento, CA. 27-31 July. 2002. (Available on-line at http://www.naaic.org/Meetings/National/2002meeting/2002Abstracts/Shearer.pdf.) (verified 7 Nov. 2002.)

Tabe, L.M., T. Wardley-Richardson, A. Ceriotti, A. Aryan, W. McNabb, A. Moore, and T.J.V. Higgins. 1995. A biotechnological approach to improving the nutritive value of alfalfa. J. Anim. Sci. 73:2752-2759.

Tesfaye, M., S.J. Temple, D.L. Allen, C.P. Vance, and D.A. Samac. 2001. Overexpression of malate dehydrogenase in transgenic alfalfa enhances organic acid synthesis and confers tolerance to aluminum. Plant Physiol. 127:1836-1844.

Thomas, J.C., C.C. Wasmann, C. Echt, R.L. Dunn, H.J. Bohnert, T.J. McCoy. 1994. Plant Cell Reports. 14:31-36.

Tuboly, T., W. Yu, A. Baily, L. Erickson, E. Nagy. 2000. Development of oral vaccine in plants against transmissible gastroenteritis virus of swine. *In* J.P. Toutant and E. Balazs (eds.) Molecular Farming. Proceedings of the OECD Workshop, La Grande Motte, France, 3-6 September 2000. p.239-248.

Winicov, I. 2000. Alfin1 transcription factor overexpression enhances plant root growth under normal and saline conditions and improves salt tolerance in alfalfa. Planta. 210:416-422.

Winicov, I., and C.R. Bastola. 1999. Transgenic overexpression of the transcription factor Alfin1 enhances expression of the endogenous MsPRP2 gene in alfalfa and improves salinity tolerance of plants. Plant Phys. 120:473-480.

Xie, D., S.B. Sharma, N.L. Paiva, D. Ferreira, and R.A. Dixon. 2002. *BANYULS* encodes anthocyanidin reductase active in plant flavonoid biosynthesis. Science (in press).

Ziegelhoffer, T., J. Will, and S. Austin-Phillips. Expression of bacterial celluslase genes in transgenic alfalfa (Medicago sativa L.), potato (Solanum tuberosum L.), and tobacco (Nicotiana tabacum L.). 1999. Mol. Breed. 5:309-318.