Peggy Lemaux

BIOTECHNOLOGY IN ALFALFA

Heather A. Koshinsky and Peggy G. Lemaux[1]

ABSTRACT

A major challenge for agriculture in the 21st century is to continue to provide food and feed for humans and animals while protecting the environment through improved resource utilization and lower input of agrichemicals. The ability to achieve these goals will depend in part on the coupling of classical methods for the manipulation of crop plants with the newer methods of genetic engineering. Biotechnological approaches can lead to increases in crop quality, pest and disease resistance and value-added potential. The biotechnological cornerstones of being able to culture plant cells *in vitro* to achieve plant regeneration and to introduce new genes stably into cells have been established in alfalfa. To date, several strategies have been used to improve alfalfa, including those for insect, viral and fungal resistance. The most successful of these appears to have been the introduction of a gene which confers resistance to *Phytophthora.* Considerable effort has also been focused on the use of alfalfa for making novel value-added products, such as pharmaceuticals and industrial enzymes which have the potential for increasing the value of alfalfa. Basic research is being conducted to develop effective strategies for engineering other important traits in alfalfa, including stress tolerance and nitrogen assimilation.

Key Words: **Biotechnology, genetic engineering, pest/disease resistance, agronomic improvement, value-added products**

INTRODUCTION

Most modifications aimed at improving crop plants focus on increased stress resistance (disease, pest, abiotic), modified nutritive or processing value or increased yield. Many of these modifications have been the objective of traditional breeding methods. However, sometimes classical methods are inadequate or the desired trait is unavailable in closely related germplasm. In these cases biotechnological methods can offer alternatives or adjuncts. In addition, in situations where plants can be used to produce non-conventional products, such as vaccines,

[1] H. Koshinsky, Postdoctoral Fellow, USDA-ARS-Plant Gene Expression Center, 800 Buchanan Street, Albany, CA 94710; P. Lemaux, Cooperative Extension Specialist, Department of Plant and Microbial Biology, 111 Koshland Hall, University of California, Berkeley, CA 94720.

plastics and pharmaceuticals, biotechnological methods may provide the only means to achieve the goal.

One of the cornerstones of biotechnology in plants is the ability to culture plant tissue *in vitro* and thereby regenerate an entire organism from a small piece of tissue or a single cell. This process, termed totipotency, makes genetic engineering in plants easier than in animals since in animal systems individual cells or tissues cannot give rise to an entire organism. The ability to culture single cells *in vitro* to give rise to entire plants provides the opportunity to select for natural mutants from or to introduce new genetic material into cultured cells, either of which can lead to plants with improved characteristics.

in vitro TISSUE CULTURE IMPROVEMENT METHODS FOR ALFALFA

During the *in vitro* culturing process a plant tissue, the source of which varies with different plant species, is isolated, sterilized, induced with hormones to form undifferentiated tissue, called callus, and finally triggered to form shoots and roots. The first demonstration of the ability to regenerate an alfalfa plant from undifferentiated tissue, in this case from stem tissue, occurred in 1972 (Saunders and Bingham, 1972). Although in many plants undifferentiated tissue will not form plants after *in vitro* culturing, alfalfa can be regenerated from callus tissue formed from a variety of plant tissues, including protoplasts, plant cells from which cell walls have been removed. As with many other plant species, however, it was found that plants could not be regenerated from all cultivars of alfalfa (Christou, 1992). In alfalfa regeneration tends to be less efficient in commercial varieties than in those genotypes used in the laboratory. This inability to manipulate all cultivars *in vitro* hinders the direct improvement of commercially important cultivars and requires that introduced genes be introgressed into commercial germplasm from the manipulated cultivars. This is an area in which improvements must occur if biotechnology is to be maximally effective in providing alternatives.

The characteristics of *in vitro* growth permit the use of this phase for the selection of natural mutants with desired characteristics, this in the absence of introducing new genetic material. During the *in vitro* phase, small pieces of undifferentiated tissue are maintained on medium that provides nutrients for growth and hormones for maintaining the undifferentiated state. Chemicals can also be incorporated into the medium allowing for selection of tissues with the ability to grow in its presence. There are several examples of the use of these technologies in alfalfa. First, in Mexico cell culture methods are being used to produce salt-tolerant alfalfa (Gutierrez-Mora, 1994). Culturing *in vitro* culturing was also used to address the problem of alfalfa's being sensitive to acid soils containing high levels of aluminum. In field situations this leads to a requirement for high lime and fertilizer input to achieve high yields. Alfalfa tissues resistant to aluminum/acid stress *in vitro* were regenerated to give plants that grew in acid/aluminum soils with no signs of yellowing or lack of growth (Kamp-Glass *et al.*, 1993). Field confirmation of resistance, however, has not been reported. Non-tissue culture methods have also been used to select for acid tolerance (Dall'Agnol *et al.*, 1994); a test has been developed using hemotoxilin that permits screening for acid/aluminum tolerance in two days (D. Powell, personal communication). The use of resistant varieties is the most effective control method for *Fusarium* wilt, a significant disease problem in the southern and southwestern United States. Plants regenerated from tissues selected on culture medium containing *Fusarium* filtrate were resistant

The most common method of transforming alfalfa is *Agrobacterium*-mediated gene delivery. This strategy is based on the introduction of DNA into plant cells by a naturally occurring, gall-producing microbe, *Agrobacterium*. This organism is capable of injecting its DNA into host plant cells in order to change their synthetic capacity so that they produce compounds necessary for their growth. Scientists have exploited the capacity of *Agrobacterium* to inject foreign DNA into plant cells by removing the tumor-inducing genes and replacing them with the genes of interest. The introduced DNA becomes a heritable part of the plant genome; cells that have incorporated the new genetic information can be identified by selection for one of the introduced genes, normally for antibiotic or herbicide resistance. The transformed tissue is regenerated into plants, each cell of which contains both the selected gene and any other genes introduced at the same time. In alfalfa this transformation procedure was first successfully used in 1986 (Deak *et al.*, 1986, Shahin *et al.*, 1986), the success of which was largely due to the earlier work of Bingham on regeneration (referred to in Christou, 1992). Transformation and regeneration are now routine in some alfalfa varieties, such as RSY27 (S. Austin, personal communication).

CHANGES ENACTED THROUGH GENETIC ENGINEERING

There are a number of different objectives being pursued using this methodology. Some of these gene introductions have led to field trials. In the United States there have been 17 applications for field trials, representing plants containing eight different genes including those for insect, fungal, viral and herbicide resistance, and novel traits. None have yet reached commercialization.

Insect Resistance

The alfalfa weevil is a major coleopteran pest of alfalfa and is ineffectively controlled with pesticides. In 1994 Mycogen was granted a permit for field trials of a coleopteran-resistant transgenic alfalfa. This germplasm contained a gene for a specific insecticidal protein from the soil bacterium, *Bacillus thuringiensis*. This insecticidal protein has been used for many years by backyard gardners and organic farmers as a topical formulation to control a variety of pests. By engineering plant cells to make this insecticide the control agent is more effectively delivered to the weevil in its "meal". In 1996 Mycogen applied for but withdrew a permit for conducting field trails on lepidopteran-resistant alfalfa, based on the use of related genes from *Bacillus thuringiensis* species. The status of these lines is unknown; Mycogen was unwilling to comment .

The Noble Foundation has developed transgenic alfalfa containing the gene for the B chain of cholera toxin. Preliminary studies in transgenic tobacco indicated that the presence of this gene product induces plant defense reactions and increases pathogen resistance. The alfalfa plants are currently being evaluated at the Scripps Institute (R. Dixon, personal communication).

Herbicide Resistance

Although herbicides are generally not used with alfalfa, transgenic plants tolerant to herbicides and antibiotics have been generated because these genes are used as the selectable markers for identifying transformed lines. In 1989 Northrup King filed for a permit to field-test Basta- or glufosinate-tolerant alfalfa; however, the outcome of these tests is not known. In Belgium, Plant Genetic Systems developed Basta-resistant alfalfa, which was field-tested and found to be unaffected by the herbicide after one and two weeks post-spraying; control plants were

completely bleached (D'Halluin *et al.*, 1990). In Australia, the Genetic Manipulatory Advisory Committee, which regulates release of transgenic organisms in Australia, has decided that the herbicide-resistance traits often used as selectable markers during transformation are undesirable in alfalfa because it is an outcrossing, perennial pasture species. For this reason, the use of the antibiotic resistance has replaced herbicide resistance as a selectable marker in the generation of transgenic alfalfa in Australia (L. Tabe, personal communication).

Viral Resistance

Alfalfa mosaic virus (AMV) is transmitted through aphids, with alfalfa grown in temperate climates generally being more severely affected than that in colder areas. One effective method to engineer resistance to viruses involves the introduction into plant cells of the coat protein gene of the target virus. Through a mechanism that is still not fully understood, the presence of this gene prevents the replication of the virus and thereby its spread and symptoms. Researchers at Pioneer Hi-Bred introduced the AMV coat protein gene into alfalfa and demonstrated significant AMV resistance in the greenhouse. The gene was crossed into dormant and non-dormant elite lines; however, in international field trials the transgenic lines did not show higher yield or reduced AMV encroachment. Additionally the trait appeared to "degrad"; lines were AMV-resistant the first year but significant AMV encroachment occurred in the second year. Pioneer will not be marketing these transgenic AMV-resistant varieties nor do they plan to pursue AMV resistance in the future through transgenic methods (T. Woodward, personal communication). In Australia, CSIRO has developed transgenic alfalfa lines containing the AMV coat protein that are currently being evaluated (L. Tabe, personal communication).

Fungal Resistance

Phytophthora root rot is one of the most significant diseases of alfalfa (Christou, 1992). Transgenic alfalfa plants were generated that contain gene(s) for an enzyme(s) that degrades fungal cell walls. In greenhouse studies transgenic lines with the glucanase gene were resistant to *Phytophthora megasperma*; lines with a chitinase gene were not. Neither gene provided resistance to infection by a fungal pathogen with large amounts of chitin in its cell walls, *Stemphylium alfalfae* (Masoud *et al.*, 1996). In 1996 the Noble Foundation received a permit to conduct field trials of transgenic fungal-resistant alfalfa containing the glucanase gene (R. Dixon, personal communication).

Crop Quality

Rumen supplements of sulfur-rich proteins can increase wool growth rates in sheep. At CSIRO in Australia, a gene encoding a sulfur-rich protein from sunflower was introduced into alfalfa to improve its quality. The amount of the sunflower protein that accumulated, however, was below that believed to be needed to increase wool growth rate in sheep (Tabe *et al.*, 1995).

The annual *Medicago* species are an underexploited resource for the improvement of alfalfa (J. Saunders, personal communication). The annual species, *Medicago trancatula*, has significantly different growth habit, pod production and seed size compared to other annual species (Diwan *et al.*, 1994). Researchers at the Noble Foundation introduced the genes for Basta tolerance into this species to demonstrate transformation capability (R. Dixon, personal communication).

Kanamycin resistance was used to demonstrate the transformation of the annual, *Medicago varia*, which has enhanced embryogenic potential (Chabaud *et al.*, 1988).

"Pharming"

The potential also exists to use alfalfa as a bioreactor or pharming crop to make products of high value through the introduction of novel genes. The characteristics which make alfalfa attractive for this purpose are that it (a) is a perennial, (b) fixes nitrogen, (c) conserves soil, (d) has relatively few pests and (e) produces large quantities of biomass. Additionally, technologies exist for the production of protein juices from alfalfa, thereby facilitating purification of value-added products, and for the marketing of the byproducts as feed and food supplements (D. Putnam, personal communication). Calculations based on the worth of both the value-added product and the by-products suggest that this type of alfalfa would be at least eight times more valuable than alfalfa hay (Austin and Bingham, 1996). Numerous novel proteins have been introduced into alfalfa. In Russia, alfalfa plants have been developed that contain the human -interferon gene (Smolenskaja *et al.*, 1994). In Canada alfalfa plants have been developed that contain both the light and heavy chains of human antibodies and the accumulation and characterization of the mature proteins is being studied (Khoudi *et al.*, 1994). The University of Wisconsin has developed transgenic alfalfa lines containing -amylase to be used in starch-processing and lignin peroxidase to be used in biopulping and bioleaching (Austin and Bingham, 1996). In field tests, production of lignin peroxidase appeared deleterious; the plants had reduced growth and development, yellowed leaves and lower yields (S. Austin, personal communication). Transgenic plants producing -amylase performed well in field trails; however, based on enzyme activity only 0.01% of total soluble protein was -amylase. Field studies continue with these plants.

A collaborative project involving DOE, USDA-ARS, the University of Minnesota and the Minnesota Valley Alfalfa Producers is aimed at using alfalfa biomass to generate electricity, thus allowing energy production to occur with less environmental degradation. This process can be coupled with the potential use of alfalfa as a bioreactor. In this scheme, the stems can be used to produce electricity through newly developed biomass gasification systems, the leaf meal used as a high-quality animal feed, the protein juice used for production and extraction of novel products, and the growth of alfalfa used to refurbish the soil. For economic profitability, alfalfa grown for electricity production would be harvested only twice a year. Alfalfa grown this long would tend to be tall, disease-susceptible, have fewer lower leaves and have decreased feed-value due to a decrease in crude protein content after flowering. To minimize these problems, the gene for isopentenyl transferase that causes reduced apical dominance, more branching, later flowering and longer leaf-hold was introduced into alfalfa from tobacco. The characteristics of these transgenic alfalfa lines are currently being determined (D. Samac and C. Vance, personal communication).

Phytoremediation

Using plants to remove contaminants from soil and water, a process, termed phytoremediation, is currently being pursued as an environmental strategy. Alfalfa has the advantage of being a perennial crop that would not have to be replaced yearly in order to perform this function and it has deep root systems which could reach groundwater reservoirs. Work has been initiated to engineer alfalfa to degrade atrazine, a contaminant from agricultural run-off frequently found in groundwater in the United States. A bacterial gene responsible for atrazine degradation is being

introduced into alfalfa with the hope that its expression in alfalfa roots will lead to detoxification of atrazine in the groundwater (C. Vance, personal communication).

Nitrogen Fixation

The possibility also exists to alter crop performance by changing parameters other than the genetics of the crop itself. With nitrogen-fixing crops like alfalfa, this could involve the alteration of the bacteria that are involved in nitrogen fixation. *Rhizobium meliloti* has been genetically engineered with extra copies of the genes that regulate nitrogen fixation and those required to supply the carbon and energy source for nitrogen fixation. In limited trials, when these bacteria were inoculated into the soil near alfalfa plants, there was a significant (13%) increase in alfalfa biomass compared with plants from the soil inoculated with unaltered bacteria (Bosworth *et al.*, 1994; Wacek, 1994). This bacterial strain is being commercialized by Urbana Laboratories in Missouri.

SUMMARY

Technologies exist to introduce genes into alfalfa via the new genetic technologies, although all commercially important lines cannot be engineered. Significant progress has been made in the areas of fungal resistance and the use of alfalfa as a source for value-added products. Other efforts have focused on herbicide resistance, viral resistance and the changing of agronomic properties like bloat potential, crop quality and stress tolerance characteristics. The potential exists for significant improvements in alfalfa that can be used in concert with classical changes and strategies. There are many alfalfa traits that can be considered for possible improvement through genetic engineering technologies, including disease resistance, stress tolerance, altered forage quality, and pest/weevil resistance (Christou, 1992). In a small informal survey of persons involved with alfalfa in the United States, the changes in order of desirability would be:

Weevil resistance, anthracnose resistance, and improved lignin digestibility;
2. Fungal resistance, reduced bloat problems, improved protein quality, and resistance to damage by both animal and farm equipment trafficking;
3 Acid/aluminum tolerance, bacterial disease resistance, AMV resistance, leaf retention, and regenerability of elite lines;
4 Improved nodulation and abiotic stress tolerance including frost, cold, salt and drought; and
5 Improved water usage.

The precise disease, pest and stress-tolerance traits vary depending on the particular growing area. While water usage is not currently a problem (except perhaps in California), it is likely to become more important in the future. In California the most desirable alterations were weevil-resistance and improved forage digestibility.

The ability to make the types of precise changes discussed above requires a detailed knowledge of the organisms and the biochemical pathways involved in the traits of interest. Therefore, much basic research is needed before these sophisticated technologies can be utilized to address some specific problems. For example, in Canada the proteins involved in cold tolerance in alfalfa are being studied (Laberge *et al.*, 1994). Once the genes directly responsible for this stress tolerance

characteristic have been identified and characterized, they can be utilized to produce cold-tolerant plants. Similarly, significant effort is being expended to understand the genes involved in nitrogen assimilation in alfalfa (Vance *et al.*, 1995). Once understood, the possibility exists to manipulate them to produce alfalfa plants with better nitrogen assimilation qualities.

An important point to remember about the new genetically altered crop species is that they are in general not "magic bullets"; their most effective use will likely be in concert with other tools available to the breeder and farmer. Traditional methods of altering crops and controlling pests have worked effectively in many situations. An example is the development of leafhopper resistance in alfalfa through classical breeding strategies. This work started at Kansas State and Purdue Universities in 1985 when researchers turned diploid lines of *Medicago* with increased glandular hair production into tetraploid lines. Pioneer Hi-Bred and other companies have introduced this trait into commercial alfalfa varieties. One such variety, Pioneer 5347, will be introduced commercially in 1997 and has true leafhopper resistance, not simply a masking of the yellowing associated with an infestation (G. Board, personal communication). In many cases, genetic engineering approaches can be used to complement or extend these approaches, providing additional forms of pest resistance or additional quality traits.

One potential obstacle to the successful application of the new genetic technologies to the challenges in alfalfa stems from the fact that, unlike corn, potato or soybean, it is not possible to retain high-value proprietary seed (S. Austin, personal communication) and therefore commercial investment will be limited; development of improved lines may depend heavily on public sector efforts. Perhaps the largest obstacle to alfalfa improvement via genetic engineering lies in its inherent genetic properties. Alfalfa is an autotetraploid making it difficult to construct lines that are missing a gene or which are homozygous for a gene (C. Vance, personal communication); introgressing genes from one line into another is also problematic.

ACKNOWLEDGMENTS

The authors would like to thank S. Austin, G. Board, R. Dixon, D. Powell, D. Putman, J. Saunders, D. Samac, L. Tabe, C. Vance, and T. Woodward for personal communications which made this manuscript more than it would have been otherwise and B. Alonso for clerical assistance.

REFERENCES

Arcioni, S., E. Nenz, F. Pupilli and F. Damiani. 1992. Gene transfer into *Medicago* through somatic hybridization. In Proceedings, 34th North American Alfalfa Improvement Conference, 1992, Guelph, Canada.

Austin, S. and E.T. Bingham. 1996. The potential use of transgenic alfalfa as a bioreactor for the production of industrial enzymes. In Biotechnology and the improvement of forage legumes. McKersie, B.D. and D.C.W. Brown (eds.). CAB International.

Bosworth, A.H., M.K. Williams, K.A. Albrecht, R. Kwiatkowski, J. Beynon, T.R. Hankinson, C.W. Ronson, F. Cannon, T.J. Wacek and E.W. Triplett. 1994. Alfalfa yield response to inoculation with recombinant strains of *Rhizobium meliloti* with an extra copy of *dctABD* and/or modified *nifA* expression. Applied and Environmental Microbiology 60:3815-3832.

Broderick, G.A. 1995. Desirable characteristics of forage legumes for improving protein utilization in ruminants. J. Anim. Sci. 73:2760-2773.

Chabaud, M., J.E. Passiatore, F. Cannon and V. Buchanan-Wollaston. 1988. Parameters affecting the frequency of kanamycin resistant alfalfa obtained by *Agrobacterium tumefaciens* mediated transformation. Plant Cell Reports 7:512-516.

Cheng, J., Y.S. He, T.A. Campbell and J.A. Saunders. 1992. Pollen electroporation for gene transfer in alfalfa. In Proceedings, 34th North American Alfalfa Improvement Conference, 1992, Guelph, Canada.

Christou, P. 1992. Alfalfa (Medicago spp.). In Genetic engineering and *in vitro* culture of crop legumes. Technomic Publishing Co. Inc., Lancaster, England.

Christou, P. 1992. Genetic engineering and *in vitro* culture of crop legumes. Technomic Publishing Co. Inc. Lancaster, England.

Dall Agnol, M., J.H. Bouton and W.A. Parrott. 1992. Selection and evaluation of alfalfa germplasms tolerant to acid, al-toxic soils. In Proceedings, 34th North American Alfalfa Improvement Conference, 1992, Guelph, Canada.

Deak, M.G., B. Kiss, C. Koncz and D. Duditz. 1986. Transformation of *Medicago* by Agrobacterium-mediated gene transfer. Plant Cell Reports 5:97-100.

Deineko, E., S. Smolenskaja, M. Rivkin, O. Tseveleva, S. Peltek and Y. Shymnyi. 1992. The use of tissue culture research in alfalfa improvement in western Siberia (Russia). In Proceedings, 34th North American Alfalfa Improvement Conference, 1992, Guelph, Canada.

D Halluin, K., J. Botterman and W. De Greef. 1990. Cell biology and molecular genetics. Crop Sci. 30:866-871

Diwan, N., G.R. Bauchan and M.S. McIntosh. 1994. Engineering of herbicide-resistant alfalfa and evaluation under field conditions. Crop Sci. 34:279-285.

Hartman, C.L., T.J. McCoy and T.R. Knous. 1984. Selection of alfalfa (*Medicago sativa*) cell lines and regeneration of plants resistant to the toxin(s) produced by *Fusarium oxysporum* f. sp. *Medicaginis*. Plant Science Letters 34:183-194.

Gutiérrez-Mora, A. and B. Rodríguez-Garay. 1992. Cell selection of polyamine over-producing variants in alfalfa. In Proceedings, 34th North American Alfalfa Improvement Conference, 1992, Guelph, Canada.

Horsch, R.B., R.T. Fraley, S.G. Rogers, P.R. Sanders, A. Lloyd and N. Hoffman. 1983. Inheritance of functional foreign genes in plants. Science 223:496-498.

Horsch, R.B., J.E. Fry, N.L. Hoffmann, D. Eichholtz, S.G. Rogers and R.T. Fraley. 1985. A simple and general methods for transferring genes into plants. Science 227:1229-1231.

Kamp-Glass, M., D. Powell, G.B. Reddy, V.C. Baligar and R.J. Wright. 1993. Biotechniques for improving acid aluminum tolerance in alfalfa. Plant Cell Reports 12:590-592.

Khoudi, H., S. Laberge, G. Allard, R. Lemieux, A. Darveau and L.P. Vézina. 1992. Production of the heavy and light chains of a monoclonal antibody in transgenic alfalfa (*Medicago sativa* L.). In Proceedings, 34th North American Alfalfa Improvement Conference, 1992, Guelph, Canada.

Laberge, S., L.-P. Vézina, Y. Castonguay and P. Nadeau. 1992. Screening and engineering for stress tolerance. In Proceedings, 34th North American Alfalfa Improvement Conference, 1992, Guelph, Canada.

Latunde-Dada, A.O. and J.A. Lucas. 1988. Somaclonal variation and resistance to *Verticillium* wilt in lucerne, *Medicago sativa* L., plants regenerated from callus. Plant Science 58:111-119.

Li, Y.-G., G.J. Tanner, A.C. Delves and P.J. Larkin. 1993. Asymmetric somatic hybrid plants between *Medicago sativa* L. (alfalfa, lucerne) and *Onobrychis viciifolia* Scop. (sainfoin). Theor. Appl. Genet. 87:455-463.

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

Pioneer Hi-Bred International. Inc. 1996. Leafhopper: facts about potato leafhopper damage and resistance in alfalfa. Information pamphlet.

Saunders, J.A., C.H. Lin, B.H. Hou, J. Cheng, N. Tsengwa, J.J. Lin, C.R. Smith, M.S. McIntosh and S. Van Wert. 1995. Rapid optimization of electroporation conditions for plant cells, protoplasts, and pollen. Molecular Biotechnology 3:181.

Saunders, J.W. and E.T. Bingham. 1972. Production of alfalfa plants from callus tissue. Crop Science 12:804-808.

Shahin, E.A., A. Spielman, K. Sukhapinda, R.B. Simpson and M. Yashar. 1986. Transformation of cultivated alfalfa using disarmed Agrobacterium tumefaciens. Crop Sci. 26:1235.

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.

Vance, C.P., S.S. Miller, R.G. Gregerson, D.A. Samac, D.L. Robinson and J.S. Grant. 1995. Alfalfa NADH-dependent glutamate synthase: structure of the gene and importance in symbiotic N_2 fixation. Plant J. 8:345-358.

Wacek, T. 1992. Field trials with genetically engineered Rhizobium meliloti. In Proceedings, 34th North American Alfalfa Improvement Conference, 1992, Guelph, Canada.