Committee on the Use of Biotechnology Research in Alfalfa Improvement
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Preamble

The 2006 report was compiled from responses received from inquiries regarding biotechnology research at laboratories around the world. The report is organized geographically. The names and addresses for a contact person at each location are listed at the end of the report. These individuals are identified by asterisk (*) in the narrative. Although we attempted to contact all labs conducting alfalfa biotechnology research, we regret any omissions that may have occurred. Please inform the committee of omissions so that the next report will be complete.

United States (compiled by Charlie Brummer and Stephen Temple)

Arizona State University

Using GFP as a reporter gene, **Ilga Winicov*** and her colleagues showed that the alfalfa *MsPRP2* promoter fragment is comparable in strength to the *CaMV 35 S* promoter in heterologous gene expression in alfalfa roots. Enhanced expression from the *MsPRP2* promoter in the presence of overexpressed Alfin1 (alfalfa root specific transcription factor) indicates that Alfin1 may be limiting in root-specific gene expression. They also demonstrated that the *MsPRP2* signal sequence led to GFP secretion from roots. In all cases, GFP expression remained root specific from the *MsPRP2* promoter cassette, even in transgenic alfalfa engineered to express *Alfin1* in leaves and stems. These findings indicate the potential application of the MsPRP2 promoter for broader use in root-specific transgene expression. For details of this research see: Winicov, I. Valliyodan, B., Xue, J. and Hoober, J.K. 2004. The *MsPRP2* promoter enables strong heterologous gene expression in a root-specific manner and is enhanced by overexpression of *Alfin 1*. Planta 219:925-935.

Cal/West Seeds

Jay Sandman, David Johnson, Lauren Johnson, and **Jonathan Reich*** are evaluating the expression of various transgenes in alfalfa (*Medicago sativa*). Cal/West has focused on the production of transgenic alfalfa via *Agrobacterium tumefaciens*-mediated transformation. Alfalfa clones amenable to the transformation system have been developed from elite germplasm and subsequent conventional breeding techniques have been employed to further improve plant regenerability. Transgenic traits of interest include regulation of plant senescence and enhanced environmental stress tolerance. Cal/West continues work with Rick Amasino of the University of Wisconsin on delayed leaf senescence. Collaborative work with John Thompson and colleagues of Senesco Technologies, Inc. is underway to develop alfalfa having enhanced environmental stress tolerance. Collaborative work with Maris Apse of Arcadia Biosciences, Inc. is aimed at developing alfalfa having improved adaptation to saline soils.

Forage Genetics International

The biotechnology research program at Forage Genetics International (FGI) is under the direction of **Stephen Temple***. FGI in collaboration with Monsanto received regulatory approval for Roundup Ready alfalfa in the USA in June 2005 and subsequently in key export markets. Twenty Roundup Ready alfalfa varieties have been commercialized with one or more products adapted to every major US alfalfa market. Roundup Ready alfalfa provides an excellent weed control option for growers and represents the first genetically engineered alfalfa

trait and first perennial crop biotech trait to be commercialized. Molecular markers developed by the FGI biotechnology program and Monsanto have been instrumental in the development of the Roundup Ready experimental varieties that achieve the required high levels of trait purity. These markers and the systems developed to utilize them have overcome the unique challenges of working with an out-crossing tetraploid crop species. Additional alfalfa biotechnology research being carried out by FGI scientists is in collaboration with **Richard Dixon*, Joe Bouton*** and their colleagues at the Samuel Roberts Noble Foundation and **Neal Martin** and colleagues from the US Dairy Forage Research Center as part of the Consortium for Alfalfa Improvement (CAI). In 2005 FGI established a multi-location field evaluation of transgenic plants containing constructs designed to reduce lignin levels. The preliminary results of this study are presented elsewhere in the NAAIC proceedings. Other CAI umbrella projects where FGI has active research interests include the development of tannin and/or PPO alfalfa for improved efficiency of protein utilization. FGI is also collaborating with **Margaret Gruber***, Agri-Food Canada on specific aspects of the alfalfa tannin project.

Purdue University

Mechanisms regulating growth and stress tolerance of alfalfa continue to be the focus of **Jeff Volenec's*** program at Purdue University. Current research focuses on several themes including identifying the physiological and biochemical mechanisms controlling synthesis of vegetative storage proteins in taproots of alfalfa. These proteins are degraded and provide most of the N to shoots during their initial growth in spring and during regrowth after defoliation in summer. Other work focuses on cold acclimation and its impact on genetic differences in fall dormancy and winter hardiness. Expression of several genes is associated with improved winter survival, but the physiological role of the products of these genes remains a mystery. Finally, molecular mechanisms regulating bud dormancy and shoot development are under study. Early events in the conversion of a quiescent bud to an actively growing shoot are unknown, but could explain certain aspects of fall dormancy responses in alfalfa.

New Mexico State University

The alfalfa breeding program at New Mexico State University under the direction of **Ian Ray*** is refining its research to genetically dissect drought tolerance mechanisms in alfalfa using microarray and DNA marker technology. Two first generation backcross linkage mapping populations designed to detect drought-adaptive mechanisms were developed and used by collaborators at the Samuel Roberts Noble Foundation to construct the highest density DNA marker map currently available in tetraploid alfalfa. Experiments using *M. truncatula* DNA microarrays are being used to identify drought responsive genes in leaves and roots of a drought tolerant alfalfa cultivar. These genes are being converted into DNA markers, primarily based on single nucleotide polymorphisms (SNP). These SNP markers, and additional ones developed for targeted physiological traits, are being mapped in the previously mentioned populations. Top-cross progeny families from each BC_1 mapping population genotype were also planted in replicated field trials at Las Cruces, NM and Ardmore, OK. Forage yield under well-watered and drought-stressed conditions are being collected over multiple harvests during 2005 to 2008. Marker and phenotypic data are being compiled to identify gene specific markers that co-segregate with forage productivity under drought-stress. Our goal is to

demonstrate how genomic technologies developed in model plants can be applied to characterize and ultimately improve complex physiological crop traits.

Champa Sengupta-Gopalan*, Suman Bagga, Jose Luis Ortega, Carol Potenza and other members of the lab are working in the general area of metabolic engineering with regards to nitrogen and sulfur metabolism. The research program uses molecular, biochemical, physiological and cellular approaches. A concerted effort is being made to understand the regulatory mechanism underlying the expression of the enzyme glutamine synthetase (GS) and using this understanding to manipulate GS levels in a tissue-specific manner in transgenic alfalfa. The ultimate goal of this project is to improve nitrogen use efficiency in alfalfa and other forage legumes. Towards achieving this goal, work is also being done to understand the role of the regulatory protein, PII (C/N sensor) and the effect of over-expressing this gene in alfalfa. The project is also looking at the effect of over-expressing sucrose phosphate synthase on the expression of GS. More recently, projects have been undertaken to look at the effect of over-expressing chloroplastic glutamine synthetase on drought and salinity stress tolerance in alfalfa. Another major project in the lab is to increase the methionine content in the vegetative tissues of alfalfa to produce animal feed that is balanced in its amino acid content. The experimental approach has been to simultaneously express genes encoding for high methionine protein (corn seed storage proteins- zeins) and a gene for a key enzyme in methionine biosynthesis, cystathionine gamma synthase. Work is in progress to manipulate other key enzymes in the methionine metabolic pathway and this includes down-regulating threonine synthase and S-adenosyl methionine synthase. More recently, the lab is focusing on subjecting the different transgenic plants produced for the different projects to microarray analysis, proteomics and metabolite analysis.

The Samuel Roberts Noble Foundation, Forage Improvements Division, Ardmore OK

Zengyu Wang*, Mary Sledge*, and **Joe Bouton*** working in collaboration with Forage Genetics International and the US Dairy Forage Research Center (e.g. the Consortium for Alfalfa Improvement), are testing alfalfa lines down regulated for different genes in the lignin pathway. These lines were transplanted into the field in September 2005 under USDA-APHIS regulatory conditions and are being harvested and tissue analyzed for various nutritive quality traits.

In collaboration with New Mexico State University, an alfalfa mapping population is being evaluated under drought-stressed field environments in both New Mexico and Oklahoma in order to develop QTL markers associated with stress-responsive candidate genes and physiological/biochemical traits. This study should contribute to developing more effective strategies for improving alfalfa productivity in drought environments.

An AP2 domain-containing transcription factor gene was isolated from the model legume plant *Medicago truncatula.* One of the genes, designated *WXP1,* is able to activate wax production and confer drought tolerance in alfalfa. Over-expression of *WXP1* under the control of CaMV35S promoter led to a significant increase in cuticular wax loading on leaves of transgenic alfalfa. *WXP1* over-expression also induced a number of wax-related genes. Transgenic leaves showed reduced water loss and chlorophyll leaching. Transgenic alfalfa plants with increased cuticular waxes showed

enhanced drought tolerance as demonstrated by delayed wilting after watering was ceased and quicker and better recovery when the dehydrated plants were re-watered.

Studies were undertaken to identify quantitative trait loci (QTL) in the diploid alfalfa species for acid soil and aluminum (Al) tolerance using simple sequence repeats (SSR) markers that are useful for marker assisted selection (MAS). Three putative QTLs on LGI, LGII and LGIII explaining 38%, 16% and 27% of the phenotypic variation, respectively, were identified. In addition, a marker designed from a candidate gene known to be involved in the release of malic acid mapped near a minor QTL on LG I suggesting that this gene may be responsible for part of the variation in Al tolerance observed in this study. The SSR markers flanking these QTLs are being used for transferring them to cultivated alfalfa via marker-assisted selection and for pyramiding Al tolerance QTL.

The Samuel Roberts Noble Foundation, Plant Biology Division, Ardmore, OK

Richard Dixon*, his colleagues and numerous collaborators are currently engaged in numerous research projects targeted towards alfalfa improvement. A key project continues to be deciphering the complex networks in monolignol formation, phenylpropanoid coupling and lignin assembly with the goal of developing forage crops with improved digestibility. A major emphasis of this research was to systematically down-regulate each and every step in both monolignol formation and subsequent coupling in alfalfa. Analysis of these transgenic plants will combine proteomics and genomics with a very detailed analysis of phenylpropanoid pathway metabolites (e.g., by metabolic profiling), as well as the most comprehensive analyses of lignin macromolecular assembly undertaken thus far. This will enable identification and dissection of the various individual metabolic networks involved in forming the three monolignols (and related metabolites), and the precise effects of down-regulation on lignin macromolecular assembly. In addition to detailed chemical and biochemical analyses, plant tissues will also be evaluated to ascertain the effects on their mechanical properties. Through collaboration with **John Ralph**, US Dairy Forage Research Center, Madison, WI, various NMR techniques are being applied to provide a detailed picture of the lignin structure in the various transgenic lines generated. With **Lloyd Sumner's** group at Noble the effects of genetic modification on the cell wall proteome are being studied. Through collaboration with **Joe Noel** of the Salk Institute Structural Biology Laboratory, the potential for structure-based modification of enzyme properties for the genetic modification of lignin is being investigated. In collaboration with **Hugh Aljoe**, the effects of various different types of lignin modification on the in rumen digestibility of alfalfa forage will be determined. The Dixon laboratory was recently funded to study the relationship between lignocellulose content and composition and saccharification for bioethanol fermentation in alfalfa. This research will make use of a range of transgenic alfalfa lines down-regulated independently in each of the monolignol pathway enzymes, and screening of transposon insertion lines of *Medicago truncatula*.

Other research projects in the Dixon laboratory include: 1. Discovery of the genes involved in the control of secondary product synthesis in *Medicago* and study the genes expressed in glandular trichomes of alfalfa and *Medicago truncatula*. This research has applications for production of antimicrobials and insecticidal compounds in plants. 2. Genetic modification of health-promoting isoflavones in alfalfa. This work will allow the testing of the hypothesis that isoflavones have health beneficial effects for humans. 3. Functional genomics of triterpene saponin biosynthesis in *Medicago truncatula*. The applications of this work are to

improve the palatability and pest resistance in alfalfa. 4. Proanthocyanidin biosynthesis in *Medicago* species. The goals of this project are the development of bloat-safe alfalfa and the development of new foods with enhanced antioxidant potential for disease prevention.

South Dakota State University

Marie-Laure Sauer and **Fedora Sutton*** are conducting research to develop and express in alfalfa a vaccine against *Escherichia coli* O157:H7. The vaccine targets intimin (fused to the adjuvant LTB: heat labile enterotoxin, B subunit), a bacterial protein responsible for attachment of the bacteria to the cattle gut. Because it will be produced in alfalfa, the vaccine will be directly fed to cattle. We expect that the bacterial load in the cattle gut will be significantly diminished, thus reducing food and environment contamination with *E. coli* O157:H7 in a very cost-effective manner.

University of California, Los Angeles

The laboratories of **Ann Hirsch*** (UCLA) and **Martha Hawes** (University of Arizona) have collaborated in the successful transformation of alfalfa (*Medicago sativa*) with both sense and antisense constructs of UDP-glucuronyltransferase (UGT) to achieve a better understanding of how this enzyme functions in plants. Normally UGTs and ß-glucuronidase (GUS) function together to modulate the activity of steroid hormones in animal cells. Our previous studies using the 35S CaMV promoter demonstrated that antisensing UGT in alfalfa showed that the AS-alfalfas were severely affected in growth and also altered in their response to symbiotic rhizobia and pathogenic fungi. In more recent experiments with localizing UGT expression utilizing GUS as a reporter, we found that the pUGT-GUS construct resulted in lethality in transgenic pea, alfalfa and Arabidopsis.

We have also been using alfalfa as a model plant in our ongoing studies on developing PCR methods with the ultimate goal of verifying the integrity of dietary supplements and identifying potential contaminants or adulterants. To start, we sequenced the ITS regions of several different alfalfa cultivars and found them to be remarkably conserved. We have approximately 10 different cultivars of alfalfa growing in the UCLA Plant Growth Center.

In a study of potential contaminants present in commercial dietary supplements of alfalfa, we found that the DNA from the plant material inside the capsules was so badly degraded that we had to utilize a repair reaction to prepare the DNA for Polymerase Chain Reaction (PCR). Upon doing so, the ITS region was amplified and subjected to TOPO-cloning. Of the 20 TOPO clones sequenced, 18 of them were most similar to the ITS regions of alfalfa, but two of them demonstrated significant similarity to *Taraxacum* species (dandelion). We also utilized restriction fragment length polymorphism (RFLP) analysis to analyze for the presence of contaminants in alfalfa dietary supplements. The best matches to contaminating sequences found in the alfalfa commercial products were from *Poa hartzii*, *Erigeron annuus*, *Aster foliaceus*, *Arenaria serphyllifolia*, *Taraxacum mongolicum*, and *Echinancea paradoxa*. These contaminating species most likely were introduced into the final product when plants from alfalfa fields with co-habiting weed species were harvested. We are continuing with using alfalfa as a model plant for

developing even more sophisticated methods to determine the presence of contaminants or adulterants in dietary supplements.

Lastly, we are re-examining the effect of cytokinin, a plant hormone, on non-nodulating plant mutants. Earlier, we found that cytokinin could induce the expression of several early nodulin genes in uninoculated alfalfa roots, even in roots of the Nod$^-$ mutant MN1008. We are now comparing the responses of wild type and Nod$^-$ mutant white sweetclover (*Melilotus alba* Desr.) in response to cytokinin treatment with those of alfalfa. Both alfalfa and white sweetclover are nodulated by *Sinorhizobium meliloti*.

University of Georgia

Charlie Brummer* is continuing research on heterosis, biomass yield and composition, and stress tolerance in alfalfa at the University of Georgia, where he moved in late 2006 from Iowa State University. He is currently using SSR and SNP markers in an association mapping project to identify QTL and genes involved in biomass yield and cell wall composition in collaboration with **Ken Moore** at Iowa State University and **Jeff Doyle** at Cornell University. He is also using the *M. truncatula* Affymetrix array to identify genes associated with yield heterosis. In the near future, he will begin genetic mapping and expression analysis on aluminum tolerance and drought/heat tolerance and comparative mapping with other forage legumes, in particular white clover.

US Dairy Forage Research Center, Madison, WI

The laboratories **of Michael Sullivan*** and **Ronald Hatfield*** are using transgenic alfalfa as a model system to characterize polyphenol oxidase-mediated inhibition of post-harvest proteolysis. Because alfalfa leaves seem to have little if any polyphenol oxidase (PPO) activity or *o*-diphenol PPO substrates, alfalfa is an excellent model system for analyzing the various components involved in this enzyme/substrate system. Transgenic red clover silenced for PPO expression is also being used as a model in these studies. In collaboration with **Deborah Samac*** (USDA-ARS, St. Paul, MN) and **Richard Muck** (US Dairy Forage Research Center), transgenic alfalfa expressing a red clover PPO cDNA and PPO-silenced red clover were used to demonstrate that the abundant PPO and *o*-diphenols present in red clover leaves are responsible for decreased post-harvest protein breakdown seen for that forage crop upon ensiling. The group is currently using the transgenic alfalfa system to characterize PPO proteins with respect to substrate specificity, latency, and protein structure/function relationships; elucidate the biochemical mechanism of PPO-mediated proteolytic inhibition; and optimize the system as a practical silage treatment. To compliment the PPO work, the group is also taking both conventional and biotechnology approaches to increase *o*-diphenol production in alfalfa leaves, since lack of endogenous *o*-diphenol PPO substrates in alfalfa prevents practical use of this natural system of protein protection. A screen of the perennial *Medicago* core collection using a plate-based browning assay revealed that none of the tested accessions contain useful levels of foliar PPO or *o*-diphenols. Consequently, the group is using red clover as a model legume that accumulates large amounts of *o*-diphenols in its leaves in order to identify key enzymes and regulatory steps in the biosynthesis and accumulation of this class of compounds in leaf tissue.

USDA-ARS, Beltsville Agricultural Research Center, Beltsville, Maryland

Gary Bauchan has developed several simple sequence repeats (SSR) from three difference sources: genomic SSR (gSSR) derived from a genomic library of *Medicago sativa*; SSR derived from express sequence tag (EST) from *M. truncatula,* and bacterial artificial chromosome (BAC) derived SSR also from *M. truncatula*. These SSR have been used to separate the 11 fall dormancy check cultivars of alfalfa. Research has been initiated to identify a small number of SSR, which will be used to fingerprint alfalfa cultivars. **Andrea Skantar** with the Plant Molecular Plant Pathology Lab. has been directed to work on resistance mechanisms of alfalfa to nematodes.

USDA-ARS-Plant Science Research Unit, University of Minnesota, St. Paul, MN

Carroll Vance*, USDA/ARS, University of Minnesota in collaboration with **Deborah Samac*** and **Mesfin Tesfaye** is conducting research on the use of the Affymetrix Medicago Genechip for whole genome transcript studies of alfalfa. They have shown the Affymetrix Chip is suitable to use with alfalfa. Vance is also using the method of RNA interference (RNAi) to assess the function of genes in alfalfa roots and nodules. He has shown that alfalfa nodules have a group of calcium binding genes that are important for nodule function. Several other genes are being evaluated by RNAi. Vance in collaboration with **Bruna Bucciarelli** and Mesfin Tesfaye have also been evaluating genes that are important for phosphorous and nitrogen acquisition.

Deborah Samac's laboratory is using microarrays to identify candidate genes involved in disease resistance, aluminum tolerance, and cell wall composition in *Medicago truncatula*. The function of genes is being tested using RNAi and over-expression in both *M. truncatula* and alfalfa.

USDA-ARS, Vegetable and Forage Crops Research Unit, Prosser, WA

Richard Larsen* and **George Vandemark*** have developed PCR assays for quantifying and detecting a wide range of alfalfa pathogens, including *Aphanomyces euteiches*, *Phytophthora medicaginis*, *Verticillium albo-atrum* and *Phoma sclerotioides*. Real-time PCR assays have been employed to study the relationship between pathogen accumulation in infected plants and disease severity. In the case of *Aphanomyces euteiches*, *Phytophthora medicaginis*, and *Verticillium albo-atrum*, positive and significant correlations have been observed between pathogen DNA content in infected tissues and disease severity. These assays can provide alternative methods for selecting resistant plants, may be used to identify avirulent isolates of pathogens or quantify inoculum levels in soil, and can also be used in multiplex reactions to simultaneously select for resistance to multiple diseases or to examine the population dynamics between pathogens in mixed plant infections. **Jennifer Ariss** and George Vandemark have been exploring the use of different molecular marker systems to determine genetic relationships between alfalfa cultivars and also to examine levels of genetic diversity within cultivars. Over 600 sequence-related amplified polymorphisms (SRAPs) and amplified fragment length polymorphisms (AFLPs) markers have been detected. These markers have been used to estimate genetic relationships among the nine original sources of *Medicago* germplasm and 67 different alfalfa cultivars representing fall dormancy classes 2-10. SRAP markers appear to be very effective at separating populations into groupings that reflect fall dormancy characteristics.

Mexico (complied by Stephen Temple)

Center for Genomic Sciences. National University of Mexico

Research in **Georgina Hernández's*** group is directed to studies of carbon and nitrogen metabolism in alfalfa nodules during symbiosis with *Sinorhizobium meliloti*, a nitrogen fixing bacterium. The group has used reverse genetic approaches, such as over-expression and antisense inhibition, to modulate nodule-specific gene expression of the ammonium assimilation enzymes: glutamine synthetase (GS) and glutamate synthase (NADH-GOGAT). For these projects, both alfalfa and the model system *Medicago truncatula* are being used and some of them are in collaboration with **Carroll Vance's*** group from the University of Minnesota-USDA/ARS. Recent projects have focused in the molecular and physiological characterization of transgenic alfalfa plants with nodule specific NADH-GOGAT inhibition that resulted in drastic deleterious effects in symbiotic N/C assimilation. A long-term goal of this research is to generate transgenic alfalfa or *M. truncatula* plants improved in symbiotic N/C assimilation.

Canada (compiled by Yves Castonguay)

Agriculture and Agri-Food Canada, Saskatoon

At the Saskatoon Research Centre, Agriculture and Agri-Food Canada, **Margaret Gruber*** is continuing work on developing alfalfa with reduced bloat potential, reduced greenhouse gas production, and increased protein bypass. Her group is attempting to alter the expression of genes associated with condensed tannins in several crops. Earlier, this laboratory isolated the world's first set of *myc*-like regulatory genes, which affect the accumulation of condensed tannins in trefoil forage and in barley seed coat. More recently, the group has focused on using functional genomics tools in *Arabidopsis* and the trefoils to isolate additional novel biochemical genes with potential to modify tannin biosynthesis. One regulatory gene is being tested in alfalfa in combination with a newly-isolated tannin biochemical gene (leucocyanidin reductase) in a collaboration with Forage Genetics, International. The maize anthocyanin regulatory gene *Lc* has also been over-expressed in alfalfa, causing the reduction of flavones in forage and the accumulation of small amounts of condensed tannins in forage and developing seed. Studies in **Tim McAllister's** laboratory at the Lethbridge Research Centre indicate that the *Lc*-enhanced genotypes represent proof-of-concept that tannins in alfalfa can improve forage quality, although optimum tannin concentration has not yet been achieved. Field-grown *Lc*-enhanced forage has a lower initial rate of digestion and gas production *in vitro* compared with parental plants. Preliminary laboratory bioassays in **Julie Soroka's** entomology laboratory at SRC also indicate that *Lc*-enhanced alfalfa reduces pea aphid viability; hence, these plants are now being tested for resistance to insects under field conditions.

Agriculture and Agri-Food Canada, Ste-Foy, Québec

At Agriculture and Agri-Food Canada, in Québec City, **Yves Castonguay*, Serge Laberge, Annick Bertrand and Réal Michaud** have a number of projects on biotechnology and genomic research. Activities include: 1) Development of alfalfa populations with improved freezing tolerance and their use for the analysis of the molecular and genetic bases of superior winterhardiness. 2) Search for candidate genes polymorphism associated to cold tolerance using a bulk segregant analysis of pooled DNA samples from genotypes of alfalfa populations selectively improved for freezing

tolerance. 3) Application of the sequence-related amplified polymorphisms (SRAP) technique to uncover DNA polymorphisms associated to variation in freezing tolerance among populations recurrently selected for the improvement of that trait. 4) Analysis of the expression of key genes and of the accumulation of molecules involved in antioxydative metabolism in alfalfa populations of contrasted freezing tolerance. 5) Ongoing high throughput analysis of app. 10,0000 Expressed Sequence Tags (ESTs) isolated from cDNA libraries of cold acclimated alfalfa and functional analysis of their expression on high density grids. These ESTs have been sequenced and their expression is currently being studied using macroarray analysis. Identification of genes with potential applications in the improvement of persistence and forage quality is a priority. 6) Characterization of genes and proteins involved in proteolysis (protease and protease inhibitors) and cell wall biosynthesis (cellulase) in alfalfa in order to identify candidate genes involved in the nutritive value of alfalfa.

Medicago Inc., Quebec, Quebec

Located in Québec City, Medicago is a biotechnology company focused on the development, production and commercialization of protein-based biopharmaceutical products. Medicago has a suite of proprietary plant-based manufacturing technologies that significantly decrease the cost of producing and improving access to new biopharmaceutical drugs for both its commercial partners as well as patients. **Louis P. Vézina*** (Vice-President, Research & Development) and his team are involved in several R&D projects. Over the years, Medicago has developed a proprietary platform for the production of this new generation of products, combining natural characteristics of alfalfa with advanced genetic engineering techniques. Recent advances in expression technologies comprise the development of an efficient cold-inducible expression system and the diversification of transient expression technologies, including a virus-based expression system taking advantage of the amplification capacity of the Cowpea Mosaic Virus, a Comovirus infecting legume plants. Process research and developments have led to the installation of a downstream processing line capable of treating (extraction, clarification, concentration) up to 350 kg of plant biomass per day. Medicago has also succeeded in coupling the high flexibility and capacity of conventional plant-based technologies with the benefits of safety and control offered by confined hi-tech greenhouses. Medicago has established collaboration agreements with Acambis PLC (vaccinal proteins) and InterveXion Therapeutics LLC (antibodies for drug abuse treatment) for the development of several partner products and is also developing its own proprietary pipeline of products that includes a biogeneric product, aprotinin, as well as a cholesterol-lowering compound that is endogenous to alfalfa.

University of Guelph

At the University of Guelph, **Judy Strommer*, Pat Shewen and Reggie Lo** are testing concepts for the oral delivery of vaccines to ruminants using transgenic forages as the delivery vehicle. They are constructing chimaeric genes which encode antigenic determinants from proteins of *Mannheimia haemolytica*, a serious respiratory pathogen, and introducing them into alfalfa and clover so as to maximize their immunogenic potential. Four antigens were previously produced, all with reasonable levels of expression in RSY27. Those tested to date have been demonstrated to be immunogenic

in rabbits and to be stable in dried feed stored at room temperature. New constructs have led to exceptionally high levels of protein production in alfalfa. The resulting plants are healthy and levels of antigen are stable. Plans are underway for testing this plant material for its ability to protect calves from challenge with *M. haemolytica.*

Europe (compiled by Deborah Samac)

Dipartimento di Biologia Vegetale e Biotecnologie Agroambientali (Department of Plant Biology and Agro-Environmental Biotechnology), University of Perugia, Italy

The development of techniques to avoid the presence of selectable marker genes (SM), especially antibiotic and herbicide resistance genes, in transgenic plants could improve public perception of plant genetic engineering. To our knowledge, no alternative markers are available today for alfalfa.

Daniele Rosellini's* group is using a *Synechococcus* gene encoding glutamate 1-semialdehyde aminotransferase (GSA-AT) as SM for alfalfa genetic transformation. This gene has been shown to confer resistance to gabaculine (3-amino-2,3-dihydrobenzoic acid) in tobacco. Gabaculine is toxic to plants through a potent inhibition of tetrapyrrole compounds synthesis via binding to GSA-AT. We consider this gene a safe SM because GSA-AT is present in all plants and the encoded protein is about 73% identical to the bacterial protein. The hemL gene and the conventional SM for alfalfa, NptII, conferring kanamycin resistance, both under the control of the dual CaMV35S promoter and the Nos terminator, were introduced in the same binary vector in order to directly compare the efficiency of the two selection systems. *Agrobacterium tumefaciens* transformation of alfalfa (genotype Regen-SY1) leaf explants was performed with this vector. In two experiments, the gabaculine-based selection system showed higher efficiency. Expression of the bacterial GSA-AT gene does not appear to affect plant phenotype, and segregation is Mendelian.

A second objective of our research is the development of an efficient co-transformation protocol, whereby the useful gene and the SM are introduced in different genomic loci, so that the SM can be segregated away in the progeny of the transgenic plant. For this purpose, we have introduced the GSA-AT and the NptII genes in different binary vectors. We have selected using gabaculine, and looked for the presence of NptII (playing the part of the useful gene). We obtained kanamycin resistant plants that were crossed to an unrelated pollen parent. The segregation of the two genes is now under evaluation in the progeny.

A third objective is to isolate the alfalfa GSA-AT gene and mutate it to make the enzyme gabaculine resistant, thus obtaining a plant-derived SM. So far, we have cloned and sequenced the GSA-AT coding sequence from *Medicago sativa* and *M. truncatula.*

Istituto Sperimentale Colture Foraggere- C.R.A. Lodi, Italy

M. Carelli and Carla Scotti* are using SSR markers originating from *M. truncatula* and *M. sativa* to i) estimate the heterozygosity level of the final products of the selfing phase in alfalfa breeding method (S_2 plants selected for vigor); ii) monitor heterozygosity restoration in the crossing phase of the alfalfa breeding method; iii) estimate genetic diversity among parents of Simple, Double and Octuple hybrids and to put it into relation with hybrid performances; iv) study among and within population (ecotypes, varieties, etc.) variation by mean of bulk and single plant analyses.

In addition, a research program is being carried out in collaboration with IGV-C.N.R. Perugia, directed by **S. Arcioni** with **L. Pecetti** and **E. Piano**, on the detection of AFLP molecular markers possibly associated with the creeping-root character, a trait very difficult to deal with by conventional breeding methods, due to its erratic.

The achievement of transgenic 'marker free' plants of *M. truncatula* by using MAT vector system is the aim of the '*in vitro*' laboratory project by **M. Confalonieri**; the characterization of parental plants from breeding programs for the ability to regenerate through somatic embryogenesis is currently being carried out. Recent emphasis has been put on the genetic manipulation of saponin metabolic pathway by the expression of heterologous genes in *M. truncatula*.

A transformation program of *M. sativa* with the IPT gene controlled by the senescence specific promoter SAG12 and with the *A. thaliana* ANT gene controlled by two seed specific promoters has been developed in cooperation with IGV-C.N.R. Perugia in the framework of a project aimed at improving protein production in alfalfa.

Finally, ISCF Lodi and IGV Perugia, in the framework of a national project started in 2003, have produced collections of mutants of *M. truncatula* by insertional (T-DNA vectors containing multimerized transcriptional enhancers and retrotrasposon) and chemical mutagenesis. The screening of the collections by means of activation tagging and tilling techniques has been recently started on secondary metabolites (saponins, protease inhibitors) pathways.

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