Development of Alfalfa Varieties With Modified Carbohydrate Profile And Enhanced Stress Tolerance

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Summary

Alfalfa is a major source of protein for our livestock industries. Although it is a high value crop, it has two major limitations; namely, winterhardiness and energy content. Conventional methods, including both forage production practices and plant breeding, have not dramatically changed the energy content of alfalfa forage; therefore, a more intensive genetic approach is required to create the necessary genetic variability. This research was designed to introduce novel genes or alter the expression of existing genes in alfalfa in order to enhance the plant's ability to accumulate carbohydrates. Higher carbohydrate levels in the root/crown will provide for enhanced winterhardiness while higher levels in the herbage will provide for improved feed quality since they will be quickly available during digestion by a ruminant animal.

The research project involved both transgenic and non-transgenic approaches to modifying carbohydrates and stress tolerance. A number of specific traits were the subject of investigation including High Leaf Starch (HLS), Sucrose synthase (SS), Sucrose-phosphate synthase (SPS), Phosphofructokinase (PFP), and Heat-Shock Transcription Factor (HSF). Transgene constructs were introduced into alfalfa using either Agrobacterium or biolistic mediated transformation. Transgenic events were obtained and PCR, rt-PCR, Southern hybridizations, Western hybridizations, and enzyme analyses were used to confirm transgene expression and number of insertions. Based on field analyses, primary transgenic plants expressing high levels of the transgene were identified. These primary transgenic plants were sexually hybridized with unrelated non-transgenic alfalfa plants to produce F1 families segregating for the transgene. F1 family members were classified into two groups: those containing and those not containing the transgene. These were then evaluated in field trials at two Ontario sites, New Liskeard and Elora. Performance was measured and herbage samples were taken for quality analyses. Due to the unique carbohydrate profile, changes in carbohydrate profile were performed using enzyme assays and HPLC.

Levels of PFP activity were six-fold higher for the transgenic group compared to the non-transgenic group. Total soluble carbohydrates (TSC) and total sucrose content was higher with plants containing the Giardia PFP transgene; level of starch was not changed. Introduction of another copy of the alfalfa SS gene also modified carbohydrate profile. When the alfalfa SS was inserted in the sense orientation, starch level increased and TSC levels decreased. Conversely, when the alfalfa SS was inserted in the anti-sense orientation, starch level decreased, and TSC levels increased. These results indicated that both the Giardia PFP and the alfalfa SS transgenes can be used to alter carbohydrate profiles in the herbage.
An alfalfa Sucrose Phosphate Synthase (SPS) gene was isolated and site specific mutation employed in order to remove the regulatory phosphorylation site on the enzyme. The original and mutated SPS gene has been reintroduced into alfalfa and transgenic plants have been obtained. Evaluation of the primary transgenic plants has revealed that those containing the mutated SPS gene have much higher TSC:starch ratios in the leaves compared to the control; ratios up to 0.20 for the mutant lines compared to 0.06 in the controls. Evaluation of cold tolerance of these lines is in progress.

Heat shock transcription factors are used by cells to control a series of heat shock genes used in stress response and injury repair. An alfalfa heat shock transcription factor gene was isolated (MsHSFA4). In situ hybridization revealed that there is one locus in the genome for the MsHSFA4 gene (ie. one for each of four homologous chromosomes). Northern analyses revealed that this gene is induced in response to both cold and heat stress. Expression of transcripts of this gene increases during cold acclimation. Levels of the MsHSFA4 gene transcript in dormant cultivars (more cold tolerant) were greater in onset and in magnitude compared to non-dormant cultivars (less cold tolerant). Transgenic plants overexpressing a sense version of MsHSFA4 had greater persistence and cold tolerance compared to plants overexpressing an anti-sense version of the gene. This revealed the potential for using the MsHSFA4 as a probe to assess cold tolerance, to study acclimation, and for engineering greater stress tolerance in alfalfa.

A high leaf starch (HLS) mutant line was identified in a natural population of alfalfa. Analysis of an early generation experimental variety was conducted. The gene did not affect maturation; the maturity of the HLS line did not differ from two check varieties. Leaflet starch of the HLS line was significantly higher in the leaves at most samplings. However, TNC in the leaves, and starch and TNC in the stems, was not altered. This mutation can be used to enhance starch content in the leaf fraction of alfalfa.

From our previous work with SOD and ADH transgenes, the expression of these will enhance root mass and improve persistence in alfalfa. Genetic transformation of alfalfa with a commercial ADH3 transgene construct is underway using proprietary technologies. Selection and environmental assessment of the elite event, and quality control of the varieties developed, will require assessment of the expression of the introduced gene. As part of the research project we produced a monoclonal antibody to the introduced gene using spleen cells obtained from mice. ADH antibody producing hybridoma colonies were generated, subdivided, and were frozen. To obtain the ADH antibody, frozen cells can be thawed, cultured and ADH antibody containing media can be collected. Protocols for Western hybridization and ELISA screening of ADH transgenic populations have been developed. We are now able to routinely screen materials for the presence of a transgenic ADH protein. This will be of value for breeding, for quality control in seed production, and monitoring and tracking forage produced from an ADH transgenic crop.

Phosphofructokinase (PFP)

Phosphofructokinase plays a key role in the regulation of pyrophosphate concentration, hexose-P and triose-P pools, and stress adaptation. Its activity does not require ADP/ATP, thus does not consume energy during the conversion. A PFP gene from *Giardia lamblia* was introduced into alfalfa. This enzyme differs from plants in that it is not inhibited, or regulated, by Fructose-2,6-P_2. It was hypothesised that the introduction of this enzyme into alfalfa would improve the process of cold acclimation by facilitating carbon flow into soluble sugars to improve winter survival. A 35S-Giardia PFP construct was introduced into alfalfa via Agrobacterium mediated transformation. Twenty-two transgenic events were obtained and six plants expressing high levels of the PFP transgene were identified and each was sexually hybridized with an unrelated non-transgenic plant to produce F1 families segregating for the transgene.

Six different segregating F1 families derived from PFP transgenic plants were evaluated in field trials. Each F1 family was divided into family members (siblings) that contained the PFP transgene, and those that did not. Members of each class were planted in replicated trials at the Elora and New Liskeard Research Stations in 2001. In 2002, herbage samples were collected and analyzed for carbohydrate profile. Leaf samples had also been taken to measure PFP enzyme activity.

The PFP activity was significantly higher in siblings containing the transgene compared to siblings that did not contain the transgene. The levels of PFP acitivity were six-fold higher for the transgenic group compared to the non-transgenic group (Table 1). Total soluble carbohydrates and total sucrose content of herbage from siblings carrying the PFP transgene were higher than

those without the transgene. This was associated with numerical (but not significant) increases in sucrose (as a % of TSC) and ratio sucrose: glucose+fructose. Starch did not differ in the samples from the two groups.

Table 1. Evaluation of six alfalfa F1 families segregating for a Giardia PFP transgene. Test was established in 2001 was a randomized complete block design, four replicates, at two sites, New Liskeard and Elora ON. Herbage quality was assessed in 2002.

Class	Leaflet PFP activity (log nmol/min/mg protein +1)	Herbage content (mg/g DW)					Sucrose as % TSC	Ratio sucrose: glucose + fructose
		Starch	TSC	sucrose	glucose	fructose		
Without PFP	2.85	5.4	12.6	7.0	4.6	0.58	54.3	1.34
With PFP	4.57	5.2	13.3	7.6	4.8	0.52	55.3	1.39
LSD (0.05)	0.097	0.94	0.63	0.48	0.17	0.071	1.49	0.086

These results support our hypothesis that introduction of the Giardia PFP transgene would alter carbon flow and increase levels of total soluble carbohydrates. However, the degree of change in TSC was relatively minor considering the PFP enzyme activity was six-fold higher in the plants. This indicates that enhanced PFP in alfalfa will not by itself lead to a significant alteration in TSC content in alfalfa herbage.

Sucrose Synthase (SS)

Sucrose synthase is a major enzyme involved in sucrose metabolism. It is a bi-directional enzyme that catalyzes the interconversion of glucose + fructose and sucrose. Our hypothesis was that upregulating the activity of SS would increase the conversion of sucrose to UDP-glucose which would result in a greater accumulation of starch. An additional copy of an alfalfa sucrose synthase gene was introduced into alfalfa using Agrobacterium mediated transformation. The coding sequence was linked to a Rubisco SS promoter. The gene was introduced in both the sense and in the antisense orientation. The sense orientation was expected to increase flow of carbon to sucrose (thus accumulation of starch) while the anti-sense was expected to decrease the carbon flow to sucrose (thus reduce accumulation of starch). A series of independent transgenic events were identified and each was sexually hybridized with an unrelated non-transgenic plant to produce F1 families segregating for the sense and anti-sense transgene.

F1 families were germinated, screened for the presence of the transgene and each family separated into two groups, one group containing the gene the other group not. Replicated trials were established at the Elora and New Liskeard research stations in 2001. In 2002, herbage was collected and analyzed for carbohydrate profile.

Supporting our hypothesis, introduction of the SS gene in the sense orientation increased the level of starch, and introduction of the gene in the anti-sense orientation decreased the level of starch. (Table 2). The numeric levels of total soluble carbohydrates declined in families with the sense-SS (and increased in families with the anti-sense SS), however, these differences were not significant. There were also no significant differences in sucrose, glucose, fructose or raffinose levels.

Table 2. Evaluation of alfalfa F1 families segregating for a alfalfa sucrose synthase transgene; one transgene contained the SS sequence in the anti-sense orientation, the other contained the sequence in the sense orientation. Test was established in 2001 was a randomized complete block design, four replicates, at two sites, New Liskeard and Elora ON. Herbage quality was assessed in 2002.

Class	Herbage content (mg/g DW)					
	Starch	TSC	Sucrose	Glucose	Fructose	Raffinose
Without antisense SS	18.6	15.2	8.3	6.3	0.27	0.34

With antisense SS	17.7	14.7	7.9	6.0	0.22	0.35
LSD (0.05)	1.15	0.63	0.44	0.26	0.061	0.024
Without sense SS	12.8	14.3	7.5	6.3	0.22	0.31
With sense SS	14.2	14.6	7.6	6.5	0.24	0.32
LSD (0.05)	0.81	0.76	0.31	0.14	0.036	0.028

By introducing an additional copy of the alfalfa sucrose synthase transgene, there was an associated change in starch content (increase for the sense orientation, decrease for the anti-sense orientation). No significant differences in total non-structural carbohydrates were detected. This indicates that the modified SS activity changed the carbon flow to/from starch and that SS can be used to increase the level of starch in alfalfa herbage.

Sucrose Phosphate Synthase (SPS)

The coding sequence for an alfalfa sucrose phosphate synthase had previously been isolated. This enzyme is the major sucrose producing enzyme in plants and its activity is regulated by a reversible protein phosphorylation of Ser158. The SPS clone encodes a 1058 amino acid protein which has SPS activity when expressed in yeast. The promoter was not found to contain a TATA box; however, a GC box and a CAATT box are present. Regulatory features identified in the promoter include binding sites for transcription factor SBF-1, a binding site for ICE1, an inducer of expression under cold conditions, a CRT/DRE element common to genes expressed during cold/dehydration stresses, and a sequence element associated with light-inducible genes. These sequence elements are consistent with the proposed role of SPS in cold acclimation.

In studies of a series of alfalfa varieties, SPS leaf transcripts were found to increase after 3 hours of exposure to 4C, and after 96 hours of exposure (4 days), to maintain a steady level at approximately a five-fold higher level than was seen in the control. This effect was similar in root and crown tissue. During fall acclimation in the field, SPS transcript levels displayed an upward trend in both crown bud and root tissues, with crown bud SPS RNA levels tripling from September to December.

The alfalfa SPS coding sequence was altered using site-directed mutagenesis to remove the phosphorylation regulatory site of the enzyme. The altered alfalfa SPS gene, under control of the 35S promoter, was introduced into alfalfa in 2002/2003 using biolistic transformation. Several transgenic lines have been recovered which contain the mutant SPS (expected to be constitutively active since it was modified to prevent inactivation by SPS kinase), antisense SPS, and control lines transformed with either empty vector or no DNA. The native and antisense SPS lines do not appear to differ from the control in either starch or total soluble carbohydrate levels. However, some mutant SPS lines posses higher soluble carbohydrate: starch ratios in the leaves than are seen in the controls; up to 0.2 for mutant SPS lines vs. an average of 0.06 for the control lines. Testing to determine whether mutant SPS lines are more cold-tolerant are in progress.

Heat Shock Transcripton Factor (HSF)

A study of an alfalfa (Medicago sativa L.) class A4 heat shock transcription factor (HSF), heat shock proteins (HSP's), and an evaluation of the utility of engineering HSFs to protect against low temperature stress was conducted. To study HSFs in alfalfa, a new, cold inducible HSF homologue, designated MsHSFA4 was isolated from a cDNA library. Using northern and western macroarrays, transcript levels for HSF, HSP 18, HSP 86 and protein levels for low molecular weight HSPs (lmwHSPs) were monitored under controlled chamber and field

conditions. An Arabidopsis HSF, AtHSFA4a was engineered into alfalfa to ascertain the effects of HSF overexpression on wither survival and biomass accumulation.

Sequencing of positive clones revealed an HSF homologue that has high similarity to known class A4 plant HSFs and thus this clone was designated MsHSFA4. Sequence and structural analysis indicated that MsHSFA4 had high similarity to known class A4 plant HSF's. MSHSFA4 has several highly conserved domains and exists as a single copy gene found at two separate genetic loci. Expression in tobacco protoplasts confirmed MsHSFa4 as functional transcriptional activator that can bind and activate the promoters of heat shock genes. To measure the temperature response of MsHSFA4, alfalfa total RNA was isolated from tissues treated at 41ºC, 4ºC and tissue collected from field acclimated plants. Northern analyses revealed that the MsHSFA4-6 transcript is induced in response to heat stress, confirming its activity as a HSF.

Under both heat and cold stress, HSF, HSP 18, HSP 86 and lmwHSPs exhibited a variety of expression patterns which related to the dormancy of the plants. For MsHSFA4 expression, levels were greater in onset and magnitude in dormant cultivars as compared to non-dormant cultivars, under heat and cold stress. Under field stress, HSF, HSP 18, HSP 86 and lmwHSPs also exhibited a variety of expression patterns. Dormancy cultivar effects were observed under HSF, HSP 86 and lmwHSP expression, specifically HSP 86 expression in bud tissue, which

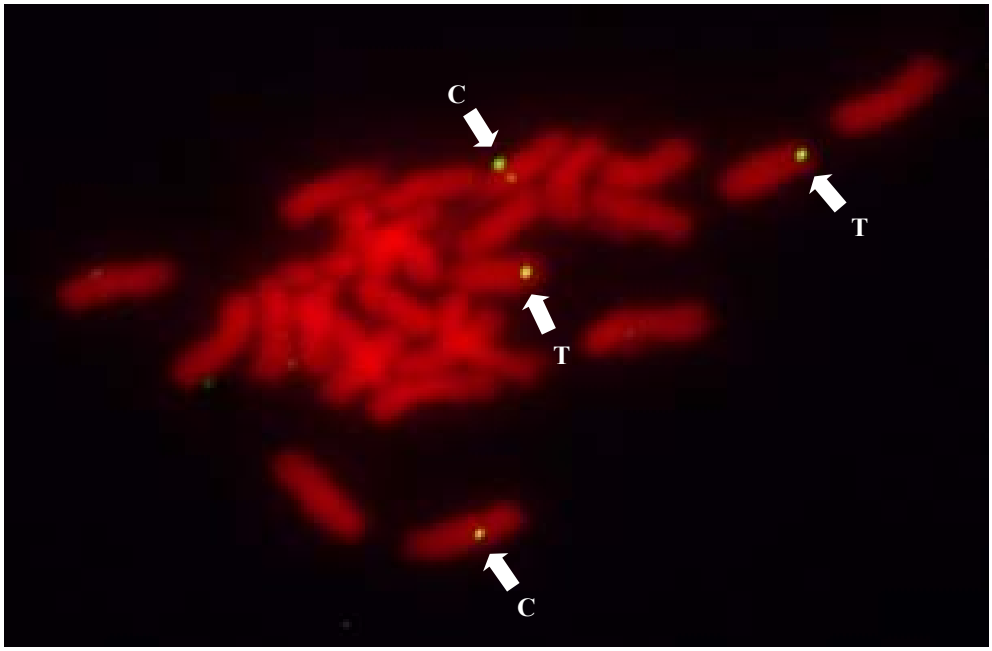

Figure 1. A digital image of a fluorescent in situ hybridization of alfalfa chromosomes isolated from root tips and hybridized using a probe for the MsHSFA4 cDNA showing four positive hybridizations (white arrows - green spots).

exhibited notably higher levels in non-dormant cultivars as compared to dormant cultivars. The results confirm the alfalfa MsHSFA4 was a functional transcriptional activator, active under heat and cold stress and involved in activation of elements of the heat shock response under low temperature stress. Engineering of AtHSFA4 in alfalfa exhibited effects on persistence and stress tolerance. Plants, and their F1 progeny, containing sense constructs exhibited increased persistence and plants containing antisense constructs exhibited decreased persistence.

This research furthered the understanding of the role of the heat shock response under low temperature by identifying the first cold responsive HSF. It offers a possible gene selective control mechanism for controlling the expression HSPs under low temperature stress. This work

has also revealed the potential for using HSFs to enhance stress protection and improve yield, with the probability of creating alfalfa cultivars with improved persistence .

GPX Promoter

Expression enhancement: A promoter, cGPX, that is responsive to oxidative stress was isolated from BAC clones from *Arabidopsis*. This promoter has a number of elements that are known to be important in other plants (ABA, desiccation, etc.). However, we do not have information to support the hypothesis that these elements are functional in a perennial legume such as alfalfa. A series of deletions in these regions were generated and some introduced into alfalfa using Agrobacterium transformation. This project formed part of a PhD thesis project. However, the graduate student has taken an extended maternity leave. Since this project was not as high as a priority as other activities in this research project, work on the GPX promoter analysis was abandoned.

High Leaf Starch (HLS)

We have identified a naturally occurring trait in alfalfa that confers a high leaf starch (HLS) phenotype. The mutation affects invertase activity in the pallisade layer of leaves. The cells have a reduced ability to export sucrose. As a consequence, starch will accumulate in the pallisade cells. The expression of the trait is specific to the pallisade layer of the leaflet. Following a backcross breeding program a set of experimental varieties were produced in 2000 and Syn-2 seed in 2001 for use in field trials in 2002. Further selections and intercrosses have continued in the breeding program.

The first experimental variety (A3300) was seeded in 2002 at two sites, Elora and New Liskeard, ON. Included in the test were two alfalfa varieties 5312 and Mangum IV. Herbage samples were taken over the first growth of the 2003 season. There was no significant difference in maturation of the three varieties (Figure 2). A3300 was numerically faster in maturation compared to the other two, but this difference was not significant.

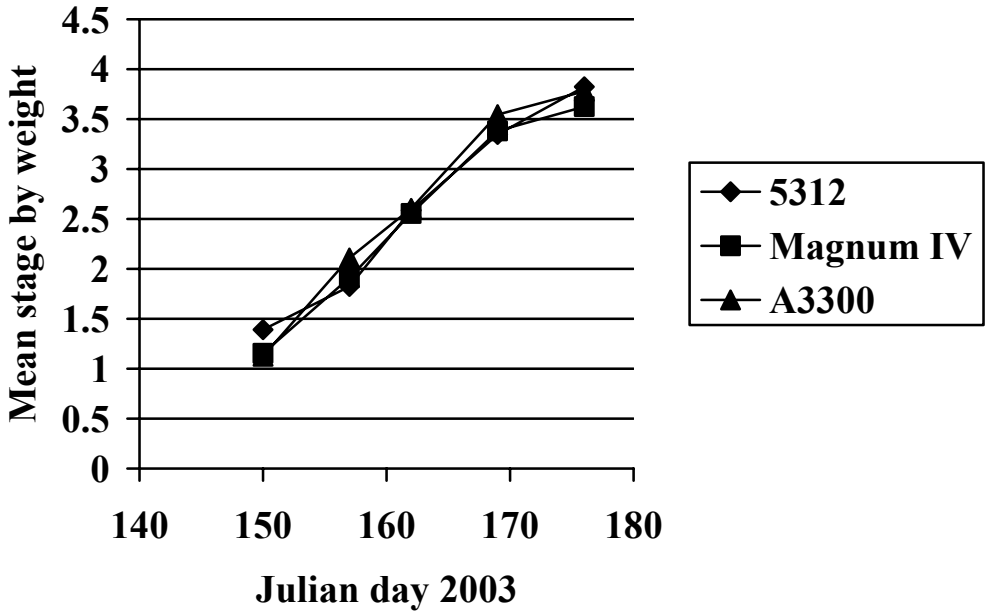

Figure 2 Mean stage by weight of three varieties of alfalfa over the period 30 May and 25 June 2003. Trial design was a randomized complete block, with four replicates, se=0.17.

Quality profile of the three alfalfa varieties is presented in Table 3. Leaflet starch was signifcantly higher in A3300 at most samplings. However, the total non-structural carbohydrates of the leaves, and starch and TNC levels of the stem did not differ. This invertase mutation alters starch content of the leaflet, but does not affect starch or TNC in other parts of the herbage.

Table 3. Carbohydrate content in leaves and stems of three alfalfa varieties at Elora, ON over the period 6 June to 25 June 2003. Test was seeded in 2002, design was a randomized complete block with four replicates.

	Starch mg/g DM Julian day			TNC mg/g DM Julian Day		
	157	162	169	157	162	169
Leaf						
5312	11.7	20.7	63.7	6.6	5.4	9.2
Magnum IV	10.0	27.8	79.1	6.1	6.0	11.3
A3300	26.6	34.9	112.5	8.5	5.9	10.7
se		9.37			0.97	
Stem						
5312	5.5	5.5	4.9	24.6	22.8	21.6
Magnum IV	7.3	6.4	7.3	32.9	23.1	24.5
A3300	6.5	5.3	8.1	34.9	19.0	17.4
se		2.33			3.83	

Antibody to an Arabidopsis ADH transgene

From our previous work with SOD and ADH transgenes, the expression of these will enhance root mass and improve persistence in alfalfa. Genetic transformation of alfalfa with a commercial ADH3 transgene construct is underway using proprietary technologies. Selection and environmental assessment of the elite event, and quality control of the varieties developed, will require assessment of the expression of the introduced gene. As part of the research project we produced a monoclonal antibody to the introduced gene using spleen cells obtained from mice.

The ADH sequence used in alfalfa transformation is a genomic sequence, however, a cDNA sequence is required for monoclonal antibody production. To generate an ADH cDNA, primers were designed for the 5' and 3' ends of the ADH gene (A1779mod and 5343). RNA was extracted from transgenic alfalfa containing ADH. RT-PCR was performed and an ADH cDNA was successfully generated. The ADH cDNA was cloned into a TOPO vector (pCR2.1-TOPO) and the sequence was checked by PCR, restriction digest and by DNA sequencing. The ADH cDNA was then cut out of the TOPO vector and cloned into an expression vector (pTrcHisA). The vector was transformed into E.coli and ADH protein was overexpressed. A time course (using SDS PAGE) was performed to verify the accumulation of ADH protein, and to determine the optimal time to harvest the protein. Western hybridization was also performed to verify the immunogenicity of the overexpressed ADH protein. Recombinant ADH protein was then isolated using a histidine column and injected subcutaneously into Balb C mice. Two injections

were performed two weeks apart, followed by an interperitoneal boost. Mice were then sacrificed and the spleen and thymus were removed. Spleen cells were fused with NS-1 myeloma cells using PEG, to create hybridomas and these were fed with thymus cells. HAT selection was carried out to select for viable hybridomas and the colonies were screened with ELISA to verify production of ADH antibody. Hybridoma colonies were then cloned by limited dilution until single colonies could be isolated. Colonies were continually screened by ELISA and Western hybridization to verify the production of ADH antibodies. Colonies were also frozen at several steps to provide back-up material. Once single, ADH antibody producing colonies were isolated, they were continuously cultured and a large amount of antibody containing media were collected. When necessary, frozen hybridoma cells can be thawed, cultured and more ADH antibody containing media can be collected.

Western hybridization was performed on transgenic alfalfa overexpressing ADH, using the ADH antibody containing media collected from the single hybridoma colonies. Several transgenic plants overexpressing ADH were identified. To test whether concentrating the antibody would identify transgenic plants overexpressing ADH at a lower level, the ADH antibody containing media was concentrated using ammonium sulfate precipitation. One additional plant expressing ADH at low level was identified by Western hybridization using the concentrated antibody.

In order to obtain a numerical value for ADH transgenic protein content and to facilitate screening large numbers of plants, ELISA was also successfully established and optimized using total protein from alfalfa and the concentrated ADH antibody. Using the ADH antibody, we are able to routinely screen materials for the presence of a transgenic ADH protein. This will be of value for breeding, for quality control in seed production and monitoring and tracking forage produced from an ADH transgenic crop.

Promotional Activities

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