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Expression of UDP–Glucose Dehydrogenase Reduces Cell-Wall Polysaccharide Concentration and Increases Xylose Content in Alfalfa Stems

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Abstract

The primary cell-wall matrix of most higher plants is composed of large amounts of uronic acids, primarily D-galacturonic acid residues in the backbone of pectic polysaccharides. Uridine diphosphate (UDP)–glucose dehydrogenase is a key enzyme in the biosynthesis of uronic acids. We produced transgenic alfalfa (*Medicago sativa*) plants expressing a soybean UDP–glucose dehydrogenase cDNA under the control of two promoters active in alfalfa vascular tissues. In initial greenhouse experiments, enzyme activity in transgenic lines was up to seven-fold greater than in nontransformed control plants; however, field-grown transgenic plants had only a maximum of 1.9-fold more activity than the control. Cell-wall polysaccharide content was lower and Klason lignin content was higher in transgenics compared to the nontransformed control. No significant increase in pectin or uronic acids in the polysaccharide fraction was observed in any line. Xylose increased 15% in most transgenic lines and mannose concentration decreased slightly in all lines. Because of the complexity of pectic polysaccharides and sugar biosynthesis, it may be necessary to manipulate multiple steps in carbohydrate metabolism to alter the pectin content of alfalfa.

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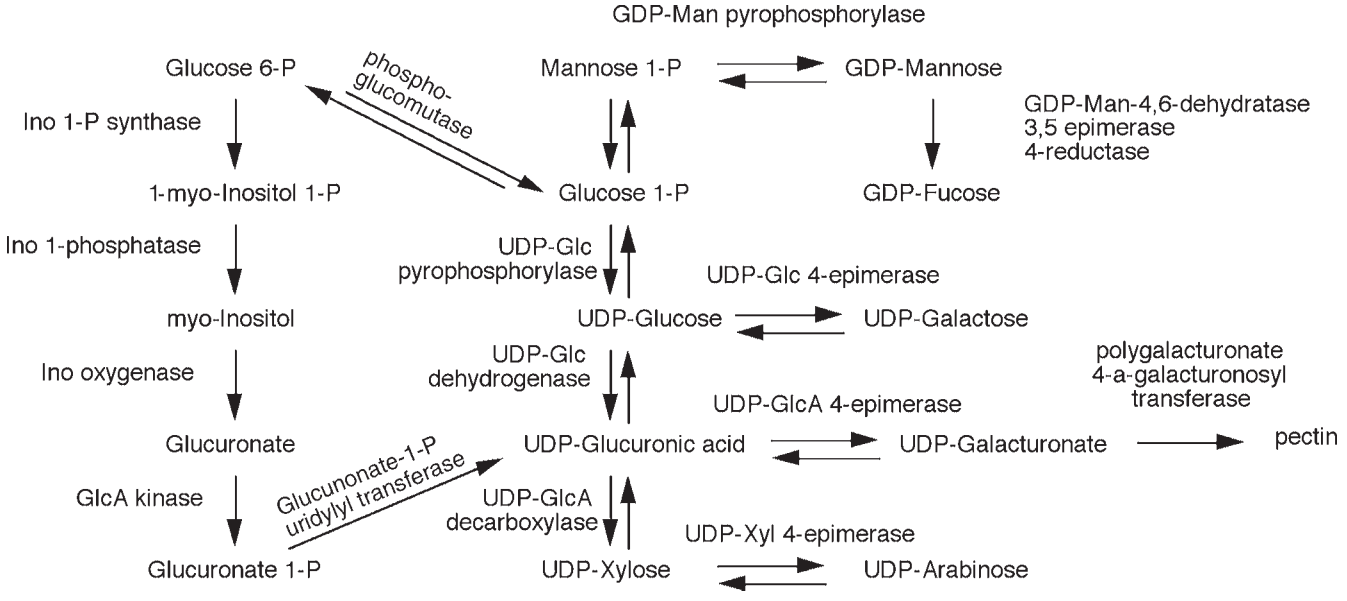

Fig. 1. Monosaccharide biosynthesis pathways.

Index Entries: Carbohydrate**;** forage; homogalacturonan; lucerne; protein utilization.

Introduction

Alfalfa (*Medicago sativa*) is an important forage crop for ruminant animals in temperate regions of the world. Forage consumed by ruminants is first digested in the rumen, where a large proportion of the nutrients is converted to microbial biomass. Remaining nutrients and the microbial biomass pass into the small intestine, where further digestion of forage material and microbes takes place *(1)*. Alfalfa leaf proteins are rapidly degraded in the rumen; however, the resulting free amino acids cannot be completely captured through bacterial growth if there is insufficient energy from carbohydrate fermentation. Although alfalfa stems contain large amounts of cell-wall carbohydrates (approx 70% of stem dry weight), the majority of the cell walls, specifically the cellulose and hemicellulose fractions, are poorly digested in the rumen. Currently, dairy producers feed large amounts of starch to partially reduce protein wastage from alfalfa. However, starch fermentation in the rumen often leads to acidosis and other health problems *(2)*.

A possible alternative to using starch-containing feeds for efficient capture of alfalfa protein is to increase the amount of pectic polysaccharides in alfalfa stems. Pectin, the major component of the cell-wall matrix, is highly digestible by ruminants with a rate of digestion similar to that of alfalfa proteins. In situ rates of pectin degradation from alfalfa range from 9% h^{-1} to >25% h^{-1}, compared with 4% h^{-1} for degradation of cellulose and hemicellulose *(3)*. The rate of pectin degradation is also faster than starch degradation (5 to 12% h^{-1}) from grains *(4)*. More important, pectin fermentation does not result in the rumen health problems associated with starch fermentation because pectin does not yield lactic acid as an end product *(5)*.

Pectin is a mixture of heterogeneous, branched, highly hydrated polysaccharides rich in galacturonic acid *(6)*. The most prevalent monomers for pectic cell-wall polysaccharides are derived from UDP–glucuronic acid (UDP-GlcA) (Fig. 1). Homogalacturonan, a homopolymer of (1→4) α-D-galacturonic acid, accounts for approx 50% of the alfalfa stem pectin *(7)*. The other pectic polysaccharides are rhamnogalacturonan-I, composed of repeated units of galacturonic acid and rhamnose with side chains predominately of neutral sugars, and rhamnogalacturonan-II, a polymer of galacturonic acid residues with heteropolymeric side chains. The primary neutral sugar in alfalfa pectin is arabinose (20–25% of the pectin), with smaller amounts of galactose, rhamnose, glucose, mannose, and fucose present. Pectin composition is relatively stable throughout alfalfa stem maturation *(7)*, supporting the general opinion that pectin is deposited in the middle lamella and primary cell wall and is not present in the secondary cell wall, which is synthesized during maturation of stem tissues.

Biochemical and developmental studies suggest that production of UDP-GlcA is a key control point in regulating the flux of UDP-monomers into pectin *(8,9)*. There is evidence for two distinct pathways leading to UDP-GlcA, which may be developmentally regulated *(10)*. However, the majority of UDP-GlcA in plant cell walls is apparently synthesized by conversion of UDP-Glc to UDP-GlcA by UDP–glucose (Glc) dehydrogenase (EC 1.1.1.22). The reaction is irreversible and apparently rate limiting *(11)*. By modifying expression of UDP–Glc dehydrogenase, it may be possible to enhance UDP-GlcA synthesis and, therefore, UDP-monomers for pectin biosynthesis.

The prime targets for enhancing pectin content in stems are xylem cells. Epidermal, collenchyma, and cortical fiber tissues in alfalfa stems develop very thick primary walls that are rich in pectin *(12)*. These thick primary walls never lignify and remain completely digestible by rumen microbes throughout stem maturation. However, xylem tissues develop lignified secondary walls rapidly and become indigestible with maturity. Because xylem tissue forms a continuous cylinder in alfalfa stems, the indigestibility of this tissue slows particle size reduction in the rumen. If increased pectin biosynthesis results in a thick, pectin-rich primary wall in xylem, then the rate of xylem tissue fragmentation and clearance from the rumen should increase. Because milk production by dairy cows is strongly related to feed intake, any increase in digesta clearance rate from the rumen should increase feed intake and animal productivity *(13)*.

Two gene promoters have been shown to direct transgene expression in alfalfa vascular tissue. In experiments to identify promoter elements involved in nodule-enhanced expression, a 536-bp sequence of the phospho*enol*pyruvate carboxylase promoter (P4) was found to be a strong promoter for expression in alfalfa vascular tissue, particularly xylem, throughout the plant *(14)*. Expression in phloem tissue was observed when an *Arabidopsis* class III chitinase promoter::GUS gene was expressed in alfalfa (unpublished). Transgenic alfalfa plants were produced containing a UDP–glucose (Glc) dehydrogenase cDNA from soybean (*Glycine max*) controlled by these vascular tissue–specific promoters. UDP–Glc dehydrogenase enzyme activity, pectin concentration, and cell-wall composition were measured for field-grown plants.

Materials and Methods

Isolation of Soybean UDP–Glc Dehydrogenase cDNA

An *Arabidopsis* cDNA library (Stratagene, La Jolla, CA) was hybridized with an *Arabidopsis* expressed sequence tag (EST)–derived probe (GenBank H36268) homologous to the previously sequenced bovine enzyme (SwissProt P12378) *(15)*, and a full-length 1732-bp cDNA (AD4) was isolated. This clone was used to probe a soybean seed cDNA library (a gift from Dr. C. Vance, USDA-ARS, St. Paul, MN) prepared from developing seed from the cultivar Lambert in the late cotyledon stage (120–160

mg/seed). Approximately 150,000 plaques were screened using the *Arabidopsis* AD4 cDNA as a probe. Screening was carried out using standard protocols *(16)*. Washes were performed at low stringency (2X saline sodium citrate, 0.1% sodium dodecyl sulfate [w/v] at 22°C). Plaques that were positive on duplicate filters were excised from the original plates and eluted in 100 m*M* NaCl; 10 m*M* $MgSO_4$; 0.01% gelatin (w/v); and 50 m*M* Tris-HCl, pH 7.5 (SM buffer) with 2% $CHCl_3$ (v/v). Positive plaques were purified three times to obtain pure preparations. Inserts were obtained by the in vivo excision method according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). Clones were submitted to the Advanced Genetic Analysis Center, University of Minnesota, for DNA sequencing.

Plant Transformation Vectors

The soybean cDNA (SD5) putatively encoding UDP–Glc dehydrogenase was modified by polymerase chain reaction (PCR) to contain an *Sma*I restriction site immediately 5' of the initiating ATG and an *Sst*I site immediately 3' of the stop codon. Primers used were SmaI-SD5 (5'-CTGCCC GGGATGGTGAAGATTTGCTGC-3') and SD5-SstI (5'-CTGGAGCTCTT ATGCCACAGCAGGCAT-3'). Reactions consisted of approx 50 pg of DNA in a 50-mL reaction with 1.5 m*M* $MgCl_2$, 50 pmol of each primer, 0.25 m*M* each dNTP, and 1 U of *Taq* DNA polymerase (Promega, Madison, WI) in the reaction buffer provided by the manufacturer. Reactions were carried out for 30 cycles of 94°C for 30 s, 55°C for 1 min, and 72°C for 1 min. The 1450-bp PCR product was purified using a QIAquick spin column (Qiagen, Valencia, CA) and cloned into pBluescript KS+ digested with *Sma*I and *Sst*I. The insert was sequenced to confirm that the coding sequence was unchanged from the original sequence. Two transformation vectors were constructed with promoters controlling the expression of SD5: an *Arabidopsis* class III chitinase promoter (Atchit) and a 536-bp portion of the alfalfa phospho*enol*pyruvate carboxylase promoter (P4). To construct the phloem-enhanced expression vector pARC205 (Atchit::SD5), the 1.5-kbp *Arabidopsis* acidic chitinase promoter was excised from pMON8896 *(17)* with *Sal*I and *Bgl*II and ligated into the *Sal*I and *Bam*HI sites of pBI101.2 to produce pARC201. The GUS coding region of pARC201 was removed by digestion with *Sma*I and *Sst*I and replaced with the modified SD5 fragment digested with *Sma*I and *Sst*I. To construct the xylem-enhanced expression vector pARC206 (P4::SD5), the modified SD5 was inserted into the *Sma*I-*Sst*I site of pBI101.2 from which the GUS gene had been removed. The *Xba*I-*Rsa*I P4 promoter fragment *(14)* was inserted into this clone digested with *Xba*I and *Sma*I. All vectors contained *nptII* controlled by the *nos* promoter for selection of transgenic plants. Vectors were mobilized into *Agrobacterium tumefaciens* strain LBA4404 by triparental mating *(18)*.

Transformation and Culture of Plants

Alfalfa leaf explants from a highly regenerable clone of Regen-SY *(19)* were cocultured with *A. tumefaciens* containing each binary vector, and

somatic embryos were produced essentially as described by Austin et al. *(20)* using kanamycin selection. After establishing plants in soil, DNA was extracted from young leaves using a Puregene Kit (Gentra, Minneapolis, MN) according to the manufacturer's instructions. Plants were tested by PCR for the presence of *nptII* using specific primers (NPTII forward: 5'-GCT ATGACTGGGCACAACAGAC-3'; NPTII reverse: 5'-CGTCAAGAAG GCGATAGAAGG-3') and subsequently for the presence of the modified SD5 cDNA using the primers *Sma*I-SD5 and SD5-*Sst*I. Reactions consisted of 1 µg of DNA in a 50-µL reaction with 2.0 m*M* $MgCl_2$, 50 pmol of each primer, 0.25 m*M* each dNTP, and 1 U of *Taq* DNA polymerase (Promega) in the reaction buffer provided by the manufacturer. Reactions were carried out for 30 cycles of 94°C for 30 s, 58°C for 1 min, and 72°C for 1 min using NPTII primers. For SD5 amplification, an annealing temperature of 55°C was used.

Because of the obligate outcrossing nature of alfalfa, all experiments were carried out using vegetative cuttings of primary transformants. Nodal stem sections were placed in sterile, moist vermiculite for approx 14 d to stimulate adventitious root production, then transferred to a mix of sand and soil (1:1 [v/v]) in 3.8 × 21 cm plastic cone-tainers (Stuewe & Sons, Corvallis, OR) and fertilized monthly with soluble 10:10:10 (N:P:K) fertilizer.

For field experiments, vegetative cuttings were made of Regen-SY, five independent lines containing the Achit::SD5 transgene (pARC205-3, -16, -17, -23, -24) and seven independent lines containing the P4::SD5 transgene (pARC206-2, -6, -8, -11, -16, -20, -22). On June 2, 1999, the plants were established in a field plot at the Minnesota Agricultural Experiment Station, St. Paul, MN. Each plot consisted of seven plants of the same line at 0.33-m intervals, with three replicate blocks. Starting July 1, 1999, plants were sprayed with Ambush (0.78 kg/ha) for potato leafhopper control at approx 15 d intervals. Plots were hand weeded and no fertilizer was applied. On July 6, plants were clipped to approx 4 cm to remove insect-damaged material. Plots were harvested on August 27, 1999 (plants in early bud), and September 28, 1999 (plants vegetative). For each plot, 15 random stems were clipped at ground level for enzyme assays. Stems were placed on ice until processed in the laboratory. Leaves and secondary branches were removed from stem samples and the stems were stored at –80°C. The remainder of each plot was clipped to approx 4 cm and the foliage dried at 55°C for 7 d and then weighed. Plots were harvested as before on June 13 and July 14, 2000, when the plants were in early flower.

RNA Extraction and Reverse Transcriptase Polymerase Chain Reaction

Total RNA was extracted from the third internode of field-grown stems from the June 13, 2000, harvest. Frozen stems were ground in liquid nitrogen using a mortar and pestle, and then the RNA was extracted using an RNeasy kit (Qiagen) as specified by the manufacturer. RNA in 50 µL of RNase-free water was treated with 1 U of RQ1 RNase-free DNase (Promega) for 1 h at 37°C according to the manufacturer's directions. RNA was puri-

fied using RNeasy columns and eluted in 100 µL of RNase-free water. Reverse transcriptase polymerase chain reaction (RT-PCR) was performed using the Access RT-PCR System (Promega) according to the supplier's instructions using 0.5 µg of RQ1-treated RNA with the primers NPTII forward and NPTII reverse with an annealing temperature of 59°C and the primers *Sma*I-SD5 and SD5-*Sst*I with a 55°C annealing temperature. The predicted products from amplification with NPTII primers and SD5 primers are 670 and 1450 bp, respectively. A 20-µL aliquot of each completed reaction was electrophoresed through 1% agarose gels in 1X TAE buffer (40 m*M* Tris-acetate, 1 m*M* EDTA), gels were stained with ethidium bromide, and bands were visualized under ultraviolet light.

Preparation of Extract and UDP–Glc Dehydrogenase Assay Procedure

UDP–Glc dehydrogenase activity was measured in stems from initial transformed plants grown in the greenhouse and in plants from the first harvests in 1999 (August 27, plants in early bud stage) and 2000 (June 13, plants in early flower stage). Extracts from field-grown plants were prepared from stem samples harvested from plants in three replicate plots. In 1999, enzyme activity was measured from the topmost three internodes of sampled stems, whereas in 2000 activity was measured from the middle three internodes of stems to decrease variation in activity owing to primary cell-wall synthesis found in younger tissues. Approximately 0.75 g of stem tissue (all stem samples were selected to have comparable diameters) was ground in a chilled mortar and pestle in 3 mL of extraction buffer (40 m*M* Tris-HCl, pH 7.5, containing 0.5 m*M* NAD^+, 1 m*M* EDTA, 2.5 m*M* dithiothreitol, 0.5% [w/v] PVPP, and 0.5 m*M* phenylmethylsulfonyl fluoride). The homogenate was filtered through cheesecloth and centrifuged at 16,000*g* for 10 min. The supernatant was removed and transferred to a new tube and centrifuged again at 16,000*g* for 10 min. The low-molecular-mass solutes were removed from the resulting supernatant by passage through an Econo-Pac10 DG column (Bio-Rad, Hercules, CA) as previously described *(21).* UDP–Glc dehydrogenase activity was measured spectrophotometrically by monitoring the appearance of NADH at 340 nm during the linear phase of the reaction. The standard assay volume was 1 mL (75 µmol of Tris-HCl, pH 8.4; 3 µmol of NAD^+; and 1 µmol of UDP-glucose). The reactions were initiated by the addition of the enzyme extract (150 µL) and monitored for 8 min. Two enzyme assays were completed per extract, and the results reported are an average of the two assays. Activity levels were calculated as previously described *(21).* Protein content was determined with Bradford reagent (Sigma-Aldrich, St. Louis, MO) according to the suppliers' instructions, using bovine serum albumin as a standard.

Analysis of Cell-Wall Components

Dried material from both harvests in 1999 was separated into leaf and stem samples. Stems were ground through a 1-mm screen in a cyclone-type

mill prior to analysis. Neutral sugar composition of the cell-wall polysaccharides was determined by the Uppsala Total Dietary Fiber procedure *(22)*, a two-stage sulfuric acid hydrolysis followed by gas chromatogrphy analysis, which measures monosaccharide composition and Klason lignin. Uronic acids were measured colorimetrically *(23)*. Based on the work of Hatfield *(7)*, alfalfa pectin can be estimated as the sum of cell-wall uronic acids, galactose, arabinose, and rhamnose. Hemicellulose sugar residues included xylose, mannose, and fucose. Glucose residues were assumed to be derived primarily from cellulose.

At the first harvest in 1999, stem internode pieces from the middle of two stems from each plot were immediately preserved in 50% ethanol. Thin sections were examined by light microscopy with and without staining for pectin to determine whether changes in the pattern of pectin accumulation occurred. Ruthenium red was used to visualize the presence of pectin in stem thin sections *(12)*.

Statistical Analyses

The field experiment was planted in three replicate blocks. Data were analyzed separately by year for enzyme activity and biomass yield. Enzyme activity data were statistically analyzed as a randomized complete block design with line as the main effect. Data for plant yield were analyzed as a randomized complete block design with line as the main effect, and harvest and its interaction with line as a split plot arrangement of treatments. Cell-wall concentration and composition data were analyzed as randomized complete block design with line as the main effect, and harvest and the line times harvest interaction as the split plot. The appropriate error terms were used to test main and split plot effects. For those traits that showed a significant *F*-test for differences among lines, individual transgene line means were compared to the nontransformed control line using the least-significant difference method. Data were considered significant at the $p < 0.05$ level. The GLM procedure in the SAS/STAT Users Guide *(24)* was used for all statistical analyses.

Results

Identification of Soybean Seed UDP–Glc Dehydrogenase cDNA

The initial screening of the soybean seed library yielded five identical partial-length cDNA clones. One of these clones was used as a probe to rescreen the library. The rescreening yielded 10 positive clones of which one contained a full-length UDP–Glc dehydrogenase cDNA. A full-length cDNA clone (SD5) was isolated that is 1738 bp long with a 71-bp 5' untranslated region, 1443-bp coding region, and 224-bp 3' untranslated region. The clone is 99.5% identical to a soybean cDNA sequence from suspension cultured cells (GenBank U53418) *(9)*. The *Arabidopsis* cDNA clones AD4 and SD5 are 72% identical at the nucleotide level and 91% identical at the amino acid level.

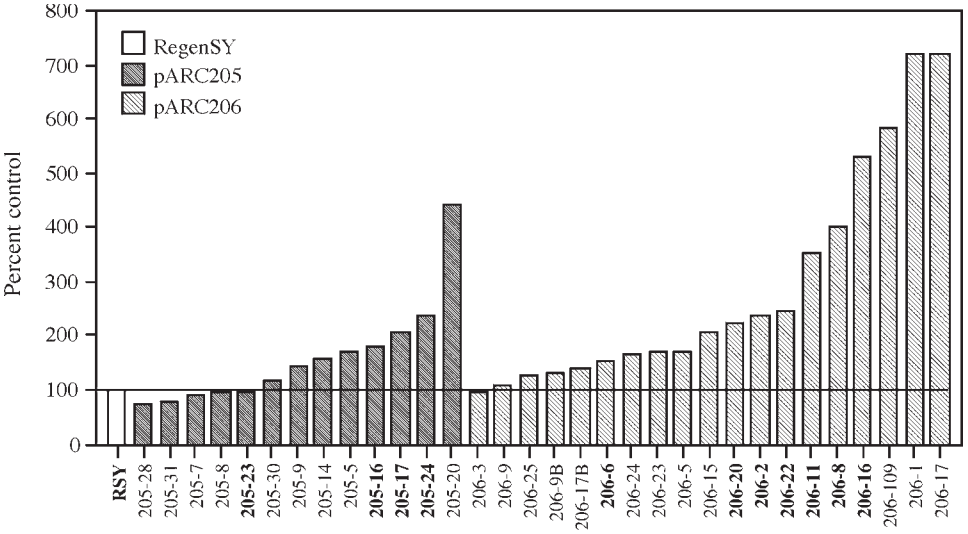

Fig. 2. UDP–Glc dehydrogenase activity in control and transgenic alfalfa plants. Stem internodes of uniform age from greenhouse-grown plants were assayed for production of UDP-GlcA. Activity is expressed as percentage of the untransformed control Regen-SY. Plant lines in bold were selected for field testing.

Selection of Transgenic Alfalfa for Field Testing

In total, 13 independent transformed plants with the Atchit::SD5 construct (pARC205) for phloem-enhanced expression and 19 plants with the P4::SD5 construct (pARC206) for xylem-enhanced expression were generated and confirmed to contain the selectable marker gene and promoter–UDP–Glc dehydrogenase cDNA constructs by PCR. Assays for UDP–Glc dehydrogenase activity were carried out twice on young plants in the greenhouse to select those with reproducibly enhanced enzyme activity for testing in the field. Control untransformed plants (Regen-SY) had a mean activity of 3.7 U $\times$ 10^{-6}/mg of protein (U = µmole of UDP-GlcA/min). Plants from 5 of the 13 lines containing the Atchit::SD5 transgene (pARC205) had activities equal to or less than the control, while 8 lines had greater activity (Fig. 2). Plants containing the transgene driven by the xylem-enhanced promoter, P4, had the greatest UDP–Glc dehydrogenase activity. In 10 lines enzyme activity was more than 200% of the activity in control plants. Based on enzyme activity and plant vigor, the following lines were selected for field testing: pARC205-16, -17, -23, -24; pARC206-2, -6, -8, -11, -16, -20, -22. Lines with the highest levels of activity (pARC205-20; pARC206-1, -10, -17) had low vigor and produced insufficient numbers of rooted cuttings for field testing.

Table 1
Enzyme Activity and Plant Biomass Yield
from Field-Grown Plants

	Control activity (%)		Yield (g/plant)[a,b]	
	1999	2000	1999	2000
Regen-SY	100	100	15.6	58.3
pARC205-16	171[d]	156[d]	4.2[d]	25.3[d]
pARC205-17	154	141[d]	6.4[d]	42.0
pARC205-23	112	122	9.1[d]	37.9
pARC205-24	87	91	5.5[d]	46.1
pARC206-6	153	151[d]	4.8[d]	44.7
pARC206-11	169[d]	109	6.2[d]	(52.2)
pARC206-16	188[d]	170[d]	5.5[d]	(24.0)
pARC206-20	151	138[d]	8.4[d]	52.0
pARC206-22	194[d]	109	4.8[d]	47.7
SEM[c]	26	13	1.2	7.4

[a] Dry matter yield averaged over two harvests each year.
[b] Yield data in parentheses were not included in statistical analysis because an insufficient number of plants survived the preceding winter.
[c] Standard error of the mean.
[d] Transgenic line was significantly different from the nontransformed Regen-SY control line ($p < 0.05$).

UDP–Glc Dehydrogenase Activity and Plant Biomass of Field-Grown Plants

Carbohydrate composition in alfalfa is strongly influenced by environmental conditions and tissue maturity. Field testing of transgenic plants provided a means to evaluate whether increasing UDP–Glc dehydrogenase activity would have a practical and quantitative impact on stem pectin content or composition in mature plants. For lines pARC206-2 and –8, an insufficient number of plants survived to assess biomass production, enzyme activity, or cell-wall composition. Table 1 shows the dry matter yield (stems and leaves) per plant for the control and remaining transgenic lines averaged across the two harvests in both years of the experiment. The line times harvest interaction was non-significant ($p > 0.05$) in either year. In the establishment year, 1999, the nontransformed control line (Regen-SY) had significantly greater biomass accumulation than the lines containing the UDP–Glc dehydrogenase transgenes. Stem biomass paralleled total biomass production (data not shown). During the second year, biomass accumulation of Regen-SY plants and six lines containing UDP–Glc dehydrogenase transgenes was similar. Data for the pARC206-11 and -16 lines could not be analyzed for the second year because insufficient plants survived the preceding winter.

The mean UDP–Glc dehydrogenase activity in Regen-SY plants in 1999 was 1.26 U × 10^{-6}/mg of protein and in 2000 the mean activity was 5.10 U × 10^{-6}/mg of protein. Because the amount of measured enzyme activity was much greater in 2000 than 1999, and the manner in which the stems were sampled varied somewhat between years, the UDP–Glc dehydrogenase activity of the transgenic lines was calculated as a percentage of Regen-SY in each year. Under field conditions, transgenic lines showed small increases in enzyme activity, compared to the control (Table 1). In 1999, the activity in lines pARC205-16, and pARC206-11, -16, and -22 (2.15, 2.12, 2.37, and 2.45 U × 10^{-6}/mg protein, respectively) was significantly greater ($p < 0.05$) than activity in Regen-SY. In 2000, significant differences from Regen-SY were observed in pARC205-16 and pARC206-6, -16, and -20 (7.96, 7.70, 8.65, and 7.05 U × 10^{-6}/mg protein, respectively).

To determine whether expression of transgenes was occurring in field-grown plants, RNA was extracted from the third internodes of stems from the June 13, 2000, harvest. RT-PCR reactions were carried out using primers specific for *nptII* and the engineered soybean UDP–Glc dehydrogenase cDNA (SD5). Reactions from all transgenic lines resulted in the expected band of 670 bp using the primers for *nptII*. Reactions from all lines containing the UDP–Glc dehydrogenase transgenes contained the expected band of 1450 bp using the primers for SD5. Reactions from the nontransformed control were negative for both PCR products (data not shown).

Stem Cell-Wall Composition

Dry matter from the first and second harvests in 1999 was separated into leaf and stem fractions to measure pectin concentration and composition in stems. Data for cell-wall concentration and composition are presented in Table 2. The results are averaged across harvests because there were no significant ($p < 0.05$) line times harvest interactions. Alfalfa stems from the second harvest were less mature with lower cell-wall concentrations (638 and 591 g/kg of organic matter; $p < 0.05$) and lower amounts of cell-wall lignin (217 and 197 g/kg of cell wall; $p < 0.05$). Averaged across the harvests, pARC205-16 and pARC206-22 had more ($p < 0.05$) cell-wall material than the Regen-SY control. Polysaccharide content of the cell walls was lower ($p < 0.05$) and Klason lignin content was higher ($p < 0.05$) in all transgenic lines compared to Regen-SY. No significant increase in cell-wall glucose, galactose, or uronic acids owing to the presence of a transgene was observed in any line. Xylose and rhamnose were consistently present in higher proportions ($p < 0.05$) in the cell-wall polysaccharides of the transgenic lines, with the exception of no difference for xylose in pARC205-17, than found in Regen-SY. This increase was relatively greater for rhamnose (36%) than xylose (15%), but in absolute terms the increase in xylose deposition in the transgenics was much greater than the increase in rhamnose. Line pARC205-17 had significantly more arabinose than the Regen-SY, and several other transgenic lines tended toward an increase in arabinose. Mannose content of the cell-wall polysaccharides was consis-

Table 2
Stem Cell-Wall Concentration and Composition of Control and Transgenic Alfalfa Plants Averaged Across Two Harvests from 1999 [a]

				Pectic sugars (g/kg polysaccharide)				Hemicellulosic sugars (g/kg polysaccharide)			
	CW (g/kg OM)	Poly (g/ kg CW)	KL (g/ kg CW)	UA	Gal	Ara	Rha	Xyl	Man	Fuc	Glc
Regen-SY	597	824	176	206	44	51	12	163	34	4.1	486
pARC205-16	640 [b]	782 [b]	218 [b]	183	42	53	16 [b]	194 [b]	29 [b]	3.6	480
pARC205-17	587	799 [b]	201 [b]	211	44	58 [b]	17 [b]	172	30 [b]	3.2	464
pARC205-23	620	793 [b]	207 [b]	190	41	54	16 [b]	187 [b]	29 [b]	3.1	479
pARC205-24	586	793 [b]	207 [b]	197	41	50	16 [b]	182 [b]	31 [b]	2.5 [b]	481
pARC206-6	627	787 [b]	213 [b]	193	41	55	16 [b]	191 [b]	30 [b]	2.7 [b]	472
pARC206-11	600	800 [b]	200 [b]	195	45	57	18 [b]	182 [b]	31 [b]	4.5	468
pARC206-16	628	771 [b]	229 [b]	193	45	54	16 [b]	184 [b]	29 [b]	3.1	476
pARC206-20	608	788 [b]	212 [b]	192	43	54	16 [b]	182 [b]	31 [b]	3.2	479
pARC206-22	654 [b]	778 [b]	222 [b]	176	39	49	16 [b]	200 [b]	29 [b]	3.3	489
SEM	15	7	7	10	1	2	0.3	5	1	0.4	8

[a] CW, cell wall; Poly, polysaccharides; KL, Klason lignin; UA, uronic acids; Gal, galactose; Ara, arabinose; Rha, rhamnose; Xyl, xylose; Man, mannose; Fuc, fucose; Glu, glucose; OM, organic matter; SEM, standard error of the mean.

[b] The transgenic line was different from the nontransformed Regen-SY control line ($p < 0.05$).

tently reduced ($p < 0.05$) in the transgenic lines by approx 12% compared to the control. Two lines (pARC205-24 and pARC206-6) had minor decreases ($p < 0.05$) in fucose content compared to Regen-SY.

No obvious differences in tissue distribution, shape, or staining for the presence of pectin were observed among the alfalfa lines. The transgenic lines appeared normal in tissue structure.

Discussion

In plants, more than 100 genes are involved in cell-wall biosynthesis. Pectic polysaccharides play a critical role in plant cell-wall structure and in plant growth and development. Although degradation of pectin, primarily owing to enzymatic activity by pathogenic microbes, has been well characterized, comparatively little is known about the process of pectin biosynthesis. Several genes encoding enzymes in the pathways for production of the monosaccharide constituents of the cell-wall matrix have recently been cloned *(25)*, paving the way for manipulating polysaccharide composition and content. Because of the central role of UDP-GlcA in pectin biosynthesis, UDP–Glc dehydrogenase appeared to be an ideal candidate for ectopic expression to manipulate the composition of alfalfa cell walls. In young, rapidly growing plants, we saw enhanced enzyme activity in a number of transgenic lines. However, enhanced activity was not retained in mature field-grown plants. The lower enzyme activity may be a result of reduced promoter activity in mature stem tissues. Although the P4 and Atchit promoters were found to be active in vascular tissue of greenhouse-grown plants, their activity in more mature tissues is not known. However, transcripts were detected in mature stems from each transgene by RT-PCR suggesting that both promoters retained activity in older stems. It is highly likely that UDP–Glc dehydrogenase is subject to feedback regulation, which would impact activity and pectin synthesis. Dalessandro and Northcote *(11)* presented evidence for feedback inhibition of UDP–Glc dehydrogenase by UDP-xylose. In addition, other enzymes involved in pectin biosynthesis are subject to feedback regulation. For example, the activity of UDP-GlcA 4-epimerase from *Streptoococcus pneumoniae* is inhibited by UDP-GalA and UDP-xylose *(26)*.

Two distinct pathways exist in plants for synthesis of UDP-GlcA, one from UDP-Glc by action of UDP–Glc dehydrogenase and an alternative pathway by way of oxidation of *myo*-inositol (Fig. 1). It is possible that UDP-GlcA synthesis in alfalfa stems occurs primarily from *myo*-inositol. However, growing evidence supports a primary role for UDP–Glc dehydrogenase in the synthesis of UDP-GlcA for primary cell-wall construction. Radioactive labeling of squash hypocotyls with ^{3}H-inositol or ^{14}C-Glc resulted in approx 1% of the label incorporated into the cell wall coming from *myo*-inositol and 40% from Glc *(27)*. Inhibitor studies with soybean callus cells showed that cell-wall synthesis continues at a normal rate when the *myo*-inositol pathway is inhibited *(28)*. Tissue-specific expression of

UDP–Glc dehydrogenase also supports a role for this enzyme in providing precursors for cell-wall synthesis. In *Arabidopsis*, UDP–Glc dehydrogenase activity occurs in root and apical meristems, young leaves, and vascular tissue of young leaves *(10).* Similarly, in soybean, expression is highest in root tips, epicotyls, and expanding leaves *(9).* Additionally, the soybean cDNA of UDP–Glc dehydrogenase from suspension cultured cells shares 82–90% DNA sequence identity with *Medicago truncatula* ESTs (GenBank AW695554, BE326183) from developing stems. The alternative pathway for synthesis of UDP-GlcA, the *myo*-inositol oxidation pathway (Fig. 1), was found to be active in *Arabidopsis* cotyledons and hypocotyls, where it may play a role in turnover of phytate *(10).*

Plants with the largest increase in UDP–Glc dehydrogenase activity (pARC205-16 and pARC206-16) appeared to accumulate less dry matter than most transgenic lines in both years, possibly owing to expression level of the transgenes. The moderate increase in UDP–Glc dehydrogenase activity in alfalfa stems led to increased xylose and rhamnose, and a decrease in mannose in the cell-wall polysaccharide fraction of most transgenic lines. Possibly by increasing concentrations of UDP-GlcA, more substrate was available for synthesis of xylose and less available for synthesis of mannose (Fig. 1). Why rhamnose content of the pectic polysaccharides increased is unclear. It appears that the pathways for monosaccharide biosynthesis are highly interconnected and that altering production of a single enzyme in this pathway may not increase the yield of such a complex end product as pectin. Dörmann and Benning *(29)* found that overexpression of the gene for UDP-Glc 4-epimerase, which catalyzes the reversible conversion of UDP-Glc to UDP-galactose, increased enzyme activity threefold in *Arabidopsis* but did not alter the ratio between UDP-Glc and UDP-galactose. Antisense expression of the gene decreased enzyme activity by 90% but did not alter the cell-wall composition. Nonetheless, pectin composition and content have been altered by single gene changes. The *mur1* mutant of *Arabidopsis* is deficient in l-fucose, a component of xyloglucan and the pectic polysaccaharides rhamnogalacturonan I and II *(30).* Pectic polysaccharide composition has also been altered by ectopic gene expression. In transgenic potato (*Solanum tuberosum* L.), expression of genes for lytic enzymes such as rhamnogalacturonan lyase led to a large reduction in galactosyl and arabinosyl residues in rhamnogalacturonan I *(31)*, and expression of endo-α-1,5-arabinanase resulted in a 70% reduction in arabinose content in tubers *(32).*

To achieve a change in the pectin content in plants, it may be necessary to redirect UDP-Glc from cellulose biosynthesis. In *Nicotiana benthamiana* transient silencing of a cellulose synthase gene caused a 25% reduction in cellulose with a concomitant increase in homogalacturonan *(33).* Possibly, by reducing cellulose synthesis, more glucose was available for pectin synthesis. An alteration in pectin composition was also observed in cell walls of the *Arabidopsis* dwarf mutant *korrigan,* which is deficient in a membrane-bound endo-1,4-β-glucanase. Cell walls of these plants have increased

amounts of homogalacturonan and a decrease in rhamnogalacturonan *(34)*. Although endo-1,4-β-glucanase is not directly involved in pectin biosynthesis, its elimination results in substantial changes in cell-wall composition, further supporting the complexity of regulation of the enzymes directing pectin biosynthesis. In both cases, alteration of pectin content resulted in aberrant cell or plant growth. Thus, constitutive changes in pectin content cannot be made without serious impacts on plant development. Most pectin in alfalfa stems is located in tissues that do not lignify during maturation such as chlorenchyma and collenchyma *(35)*. Alfalfa genotypes differing in stem pectin content have been identified (unpublished). Analysis of gene expression in these genotypes may yield clues to successful modification of alfalfa for increased pectin content and rumen digestibility.

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