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Medicago truncatula as a Model for Dicot Cell Wall Development

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Abstract We have initiated a genome-wide transcript profiling study using the model legume *Medicago truncatula* to identify putative genes related to cell wall biosynthesis and regulatory function in legumes. We used the GeneChip® *Medicago* Genome Array to compare transcript abundance in elongating versus postelongation stem internode segments of two *M. truncatula* accessions and two *Medicago sativa* (alfalfa) clones with contrasting stem cell wall concentration and composition. Hundreds of differentially expressed probe sets between elongating and post-elongation stem segments showed similar patterns of gene expression in the model legume and cultivated alfalfa. Differentially expressed genes included genes with putative functions associated with primary and secondary cell wall biosynthesis and growth. Mining of public microarray data for coexpressed genes with two marker genes for secondary cell wall synthesis identified additional candidate secondary cell wall-related genes. Coexpressed genes included protein kinases, transcription factors, and unclassified groups that were not previously reported with secondary cell wall-associated genes. *M. truncatula* has been recognized as an excellent model plant for legume genomics. The stem tissue transcriptome analysis, described here, indicates that *M. truncatula* has utility as a model plant for cell wall genomics in legumes in general and shows excellent potential for translating gene discoveries to its close relative, cultivated alfalfa, in particular. The natural variation for stem cell wall traits in *Medicago* may offer a new tool to study an expanded repertoire of valuable agronomic traits in related species, including woody dicots in the eurosid I clade.

Keywords *Medicago truncatula* · Alfalfa · Transcript analysis · Genomics · Stems

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Introduction

Plant cell wall characteristics strongly affect the availability of lignocellulosic-derived sugar for fermentation and are a major factor affecting cost and efficiency of biomass conversion to biofuels, due to the challenges of pretreatment steps [8, 34, 43]. *Arabidopsis*, as the primary model plant, has provided a research platform for important discoveries of genes and gene functions associated with primary and secondary cell wall biosynthesis. The genomic tools available for *Arabidopsis* have also been used to identify genes involved in xylem formation for application in understanding wood formation (e.g., [44, 68]). Nevertheless, it is not clear whether *Arabidopsis* will provide all the tools necessary for an expanded repertoire of agronomic traits of value in crop species. For instance, previous genetic analysis and transcript profiling studies suggest a role for specific fasciclin-like genes in both primary and secondary wall formation [39, 46, 55]. However, many fasciclins that are highly expressed during formation of cellulose-rich tension wood in *Populus* spp. appear to lack orthologs in *Arabidopsis* [2, 39].

Legumes have many traits that make them attractive bioenergy crops, especially as components of mixed grass swards or in crop rotations with maize. Alfalfa (*Medicago sativa*) is a potential bioenergy legume that fixes atmospheric nitrogen and produces leaf and stem coproducts: the leaf meal for livestock feed [14] and dried stems for conversion to syngas [15] and/or fermentation to ethanol [12]. A perennial crop with high biomass yields, alfalfa is the fourth most widely grown crop in the USA [5]. Nevertheless, studying alfalfa is challenging because it is a cross-pollinated autotetraploid, with complex segregation and inheritance patterns. Because of its ease of genetic manipulation and small genome size, barrel medic (*Medicago truncatula*) has become a model species for genomic studies of the Fabaceae, including alfalfa. In contrast to alfalfa, *M. truncatula* is a lesser-grown annual, diploid, and self-pollinating species. Comparative mapping among many legumes has shown a high degree of conservation of gene content and gene arrangement [9, 70], as well as a very high degree of DNA sequence homology between alfalfa and *M. truncatula* [60].

In previous research, four *M. truncatula* accessions and two alfalfa genotypes were evaluated for stem tissue morphology and cell wall characteristics to ascertain whether *M. truncatula* displays comparable diversity in stem cell wall traits to alfalfa [54]. One obvious morphological difference between *M. truncatula* and alfalfa plants relates to their stem growth habit. Perennial alfalfa plants each year produce erect stems, while the annual barrel medic forms decumbent stems. Nevertheless, cross sections of *M. truncatula* and alfalfa stems showed similar patterns of tissue differentiation and growth [54]. During primary growth in alfalfa, deposition of non-lignified primary walls predominates in elongating stem internodes proximal to the apical meristem. In older stem internodes of alfalfa undergoing secondary growth, synthesis of lignin- and cellulose-rich secondary wall predominate due to deposition of secondary tissues by vascular cambium [18]. During the postelongation phase, xylem vessel element and fiber cells develop lignified primary and thickened, lignified secondary walls soon after differentiation from the cambium. Phloem fiber cells also develop a thickened cellulose-rich secondary wall, but only the primary wall of phloem fibers lignifies [18]. Similarly, the range of stem cell wall composition and content among *M. truncatula* accessions was found to resemble that of alfalfa [54]. Statistically significant differences in cell wall composition among the four *M. truncatula* accessions tested indicates that naturally occurring variation in *M. truncatula* may be a rich resource for discovering mechanisms regulating cell wall biosynthesis. Overall, previously published results suggest that analysis of plant cell wall traits in alfalfa and other legumes would be facilitated by evaluation of *M. truncatula*, with well-developed genetic and genomic resources [10, 64, 67].

In *Arabidopsis*, secondary cell wall formation was shown to increase with increasing distance from the shoot apical meristem toward the base of the inflorescence stem [62]. Sampling of stem segments along this developmental gradient has been instrumental in uncovering plant genes responsible for cell wall biogenesis and control in *Arabidopsis* [7, 17]. Prassionos et al. [49] used a similar approach for sampling stem segments of hybrid aspen for transcriptome profiling. Transcript analysis of woody plants has unveiled genes involved in lignin, pectin, and cellulose biosynthesis [29, 49]. Macroarray analysis of different plant organs and stem segments has also been used to profile transcript expression patterns of cell walls in maize [26]. These efforts have uncovered many cell wall-associated genes that have putative functions in the phenylpropanoid pathway, several transcription factor (TF) gene families, cell death proteins, and transporters, among others. Additionally, proteome analysis of plant cell walls has allowed the identification of cell wall-localized proteins that have not been previously identified using transcript profiling [35, 65].

The Affymetrix *Medicago* genome array [1], which contains more than 52,000 probe sets from barrel medic and alfalfa, has been instrumental in the identification of biologically meaningful gene expression patterns in *M. truncatula* [3, 31, 60] and *M. sativa* [60]. In this study, we used the Affymetrix *Medicago* array for a genome-wide expression study in young (elongating) and old (postelongation) stem segments of the *M. truncatula* accessions A17 and DZA315.16 (hereafter referred to as DZA) and alfalfa clones 252 and 1283. These germplasms were chosen because they express divergent cell wall composition. Identification of differential expression profiles between stem developmental stages was instrumental in identifying genes with putative functions in primary and secondary cell wall biosynthesis and growth in the model legume and cultivated alfalfa.

Methods

Plant Culture

Alfalfa and *M. truncatula* plants were grown in greenhouse and controlled growth chambers, respectively. Alfalfa clones 252 and 1283, which have been identified with consistent differences in stem cell wall cellulose and Klason lignin concentrations (Lamb and Jung, unpublished), were propagated from vegetative cuttings and grown in plastic pots (10×10×10 cm) containing soil/sand (1:1; *v*/*v*) in a greenhouse. When plants reached the full flower stage of development, alfalfa plants were cut back by removing the

aerial herbage at 2-cm cutting height. Plants were allowed to regrow for approximately 6 weeks after cutting until they developed multiple stems. Stem segments were sampled at the late bud stage of development as described below. There were three replicates with 16 plants in each replicate. Plants were watered daily with tap water and fertilized weekly with water soluble fertilizer (20:10:20; N/P/K).

For the *M. truncatula* experiment, seeds of *M. truncatula* A17 and DZA were scarified with sand paper and pregerminated in Petri plates on moist Whatman filter paper for 3 days at 4°C and then moved to room temperature for 24 h. Germinated seeds with approximately equal radicle lengths were planted in pots (10×10×10 cm) containing Metro-mix 200 (Sun Gro Horticulture, Bellevue, WA, USA) and were grown in a growth chamber (light intensity of 300 μmol m^{-2} s^{-1}, temperature cycle of 25°C and 21°C, light and dark, with a 16-h photoperiod). One week after planting, seedlings were thinned to a single plant in each pot. Plants were watered with tap water as needed and fertilized weekly with water soluble fertilizer (20:10:20; N/P/K). Stem tissues were collected at 8 weeks after planting, when plants had developed multiple stems (three to four stems on each plant) with approximately eight to ten internodes per stem. There were three biological replicates with 21 pots in each replicate.

Stem Tissue Harvest

The transition from elongating to postelongation stage of stem internode development is easily identifiable in both *M. truncatula* and alfalfa by differences in pliability and suppleness of stem internodes. Stiff internodes are located lower on the stem axis and very pliable internodes near the tops of the stems. After identifying the internode which was in transition between these two developmental stages, it was excised and discarded. For microarray analysis, two internodes located immediately above (young stem segments, elongating) and below (old stem segments, postelongation) the transition internode were harvested. In general, young stem segments used for microarray analysis consisted of stem segments of the first and second internodes from the shoot apical meristem. Older stem segments generally contained the fifth and sixth internodes from the shoot apical meristem. Stem segments were immediately frozen in liquid nitrogen and stored at −80°C for subsequent RNA extraction. The remaining stem portions from each alfalfa plant (stem segments below the postelongating stem segment) were immediately collected and dried at 60°C for determination of cell wall composition [66].

RNA Extraction and GeneChip Hybridization

Approximately 150 mg of stem tissue ground in liquid nitrogen was used for total RNA extraction using TRIZOL reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. During the RNA extraction, contaminating genomic DNA was removed by incubating samples with RQ1 DNase following standard procedures suggested by the supplier (Promega, Madison, WI, USA). Ten micrograms of total RNA was used to produce biotin-labeled cRNA using Affymetrix kits following the manufacturer's suggested procedures for eukaryotic reactions (Affymetrix, Santa Clara, CA, USA). Fifteen micrograms of biotin-labeled cRNA, fragmented as suggested by Affymetrix, was hybridized to the GeneChip® *Medicago* Genome Array. The integrity and quality of total RNA and fragmented biotin-labeled cRNA were verified using the Agilent 2100 Bioanalyzer RNA 6000 Nano LabChip (Agilent Technologies, Santa Clara, CA, USA). GeneChips were hybridized, washed, stained, and scanned as previously described [60].

Microarray Data Analysis

In all of the data analyses, gene expression signals corresponding to the bacterial microsymbiont probe sets were excluded. Gene expression values were calculated with the robust multi-array average [33] using quantile normalization, as provided with the Genedata Expressionist Pro version 4.5 (Genedata, San Francisco, CA, USA). Presence or absence calls of expression data for each probe set were made using MAS5 [42]. Principal components analysis (PCA) was initially used to evaluate gene expression patterns between young and old stem internodes of *M. truncatula* and alfalfa. PCA was conducted using the Genedata Expressionist Pro version 4.5 platform (Genedata).

Statistical analysis of the stem microarray data between young and old stem segments was based on the *t* test ($p<0.05$) using the GeneSpring Expression analysis software version 7.3 (Agilent Technologies). Because the *Medicago* genome array contains a large number probe sets, *p* values were adjusted for multiple testing corrections to correct for occurrence of false positives [56]. For this, the Benjamini and Hochberg false discovery rate [4] was applied using options in the GeneSpring Expression analysis software (Agilent Technologies), and the corrected values were designated as the *q* values. Heatmaps and expression data clustering were also generated using GeneSpring Expression analysis software (Agilent Technologies). For clustering analysis, the average linkage clustering algorithm and the upregulated correlation similarity measure were used as provided in GeneSpring software (Agilent Technologies).

To identify genes that are potentially coexpressed probe sets, Pearson correlation coefficients were calculated for each probe set to identify genes that showed coregulated expression patterns with *CESA* and *COBL4* genes. For coexpression analysis, 60 publicly available *Medicago*

GeneChip data sets collected from several organs and tissues of A17 plants [3, 31], as well as the six GeneChip data sets from young and old stem segments of A17 in this study, were analyzed using GeneSpring Expression analysis software (Agilent Technologies).

The significantly differentially expressed and uniquely expressed probe sets were categorized into putative functional categories using GeneBins, an online bioinformatics tool for classifying probe sets of the *Medicago* chip [24] (http: bionfinroserver.rsbs.anu.edu.au/utils/GeneBins). Functional classifications of *Medicago* probe sets were further refined by homology searches using the predicted protein sequences of the *Medicago* chip as query sequences to perform BLASTX with an E value cutoff of 10^{-10} against plant cell wall protein families at the Purdue University cell wall genomics site (http://cellwall.genomics.purdue.edu/) and the cell wall navigator at the University of California, Riverside [23] (http://bioweb.ucr.edu/Cellwall/index.pl). For putative transcription factors, a similar sequence homology search using BLASTX with an E value cutoff of 10^{-10} was also performed against the database of *Arabidopsis* transcription factors [27] (http://datf.cbi.pku.edu.cn). Graphical display of cellular function and regulation overviews of the microarray data were oriented using the MapMan software [61] as adapted for the *Medicago* genome array [66]. All microarray data in this study have been deposited in the Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/geo/) under platform number GPL4652.

Computational and Phylogenetic Analysis

Predicted *Medicago* fasciclin gene sequences (MtFLAs) were downloaded from *Medicago* BAC sequences (www.tigr.org/tigr-scripts/medicago/IMGAG/imgag_annotator.pl?). *Arabidopsis* and poplar fasciclin gene sequences used for comparison were obtained from GenBank. Multiple sequence alignments were performed using ClustalW and phylogenetic trees were constructed using amino acid sequence alignments of full-length sequences of all predicted protein sequences using PHYLIP software [20]. Presence and location of signal peptide cleavage sites in MtFLA amino acid sequences were predicted using the SignalP 3.0 server at http://www.cbs.dtu.dk/services/SignalP/. The presence of a fasciclin-like domain was predicted using interproscan (http://www.ebi.ac.uk/Tools/InterProScan).

Results and Discussion

Chemical Composition of Alfalfa and *M. truncatula* Stems

Alfalfa clones 252 and 1283 were identified as part of a long-term breeding program for stem quality traits (Lamb and Jung, unpublished). Chemical composition data from plants grown in field plots over several growing seasons showed that stems of alfalfa clone 252 on average showed consistently higher cellulose (302±3 g kg^{-1} dry matter (DM)) and Klason lignin (165±1 g kg^{-1} DM) concentration than stems of alfalfa clone 1283 (cellulose 273±2 g kg^{-1} DM and Klason lignin 144±1 g kg^{-1} DM; Lamb and Jung, unpublished). Greenhouse grown mature stem tissues of these clones collected at the same time as the stem samples for the current microarray analysis also showed significant differences for almost all chemical composition variables evaluated [66]. As expected, stems of alfalfa clone 252 displayed significantly higher cellulose and Klason lignin concentration than stems of alfalfa clone 1283. Significant variations were also observed in cell wall uronic acids, arabinose, galactose, and rhamnose concentrations between the two alfalfa clones [66]. In alfalfa, these cell wall monosaccharides were previously shown to be the primary components of pectin [28], indicating that pectin content was considerably higher in stems of clone 1283 compared to stems of clone 252.

The two *M. truncatula* germplasms used in this study differ significantly in both stem structure and cell wall composition. Stems of A17 showed significantly higher cellulose (421 g kg^{-1} cell wall) and hemicellulose (154 g kg^{-1} cell wall) content than stems of DZA (cellulose=387 g kg^{-1} cell wall and hemicellulose=141 g kg^{-1} cell wall), while DZA stems showed significantly higher pectin content than A17 stems [54]. *M. truncatula* A17 displayed significantly longer internodes than did DZA [54], consistent with the recent report by Juliet et al. [37], who also noted a thicker stem diameter in DZA plants than stem diameter in A17 plants. In contrast, there was comparable Klason lignin content between A17 and DZA stems [54].

These four germplasms were used for identifying robust expression patterns in cell wall-related genes occurring in *Medicago* stems regardless of variation in cell wall content or stem growth patterns. The study also will serve as a baseline for later identification of genes underlying biological variation in cell wall synthesis.

Overview of Microarray Results

The Affymetrix *Medicago* genome array was utilized for global transcript profiling of young (elongating) and old (postelongation) stem segments collected from the alfalfa and *M. truncatula* plants described above. The alfalfa plants were greenhouse grown, while the *M. truncatula* plants were grown in growth chambers. Consequently, the stem microarray data were analyzed separately for alfalfa and *M. truncatula*. Signal intensity values were assessed for variability of gene expression data among the three biological replicates of each genotype. Correlation coeffi-

cients among the three biological replicates were very high, ranging from 0.94 to 0.99, providing adequate statistical power for identification of differentially expressed probe sets between young and old stem segments of alfalfa and *M. truncatula*.

Principal component analysis of the stem microarray data indicated that all three biological replicates of each genotype of *M. truncatula* and alfalfa clustered tightly together (Fig. 1a, b), substantiating the very high correlation coefficients seen among the three biological replicates. In *M. truncatula* data, the first two principal components accounted for approximately 85% of the total gene expression variation. The second principal component (PCA 2) separated two distinct clusters of data: young versus old stem segments (Fig. 1a), indicating this component represented gene expression variation based on stem developmental stages, regardless of the *M. truncatula* accessions used in the study. In alfalfa microarray data, a large proportion (82%) of the gene expression variation was also explained by the first two principal components. PCA 2 explained nearly 3% of the total gene expression variation. Along PCA 2, there were four identifiable clusters of gene expression data (Fig. 1b), suggesting that PCA 2 is a measure of gene expression variation between alfalfa clones as well as stem developmental stages.

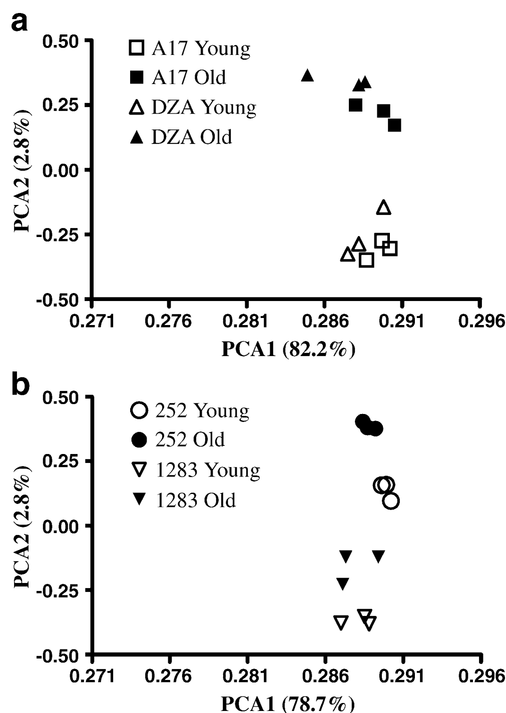

Fig. 1 Principal component analyses (*PCA*) of genome-wide gene expression data from *Medicago* stem segments. Figures represent data clustering along the first two principal components for stem microarray data for (**a**) *M. truncatula* and (**b**) alfalfa. The percentages show gene expression variation explained by each principal component

The number of probe sets detected in stem segments of the two *M. truncatula* accessions was considerably more than the number of probe sets detected in stem segments of the two alfalfa clones. The percentages of detected probe sets in stem segments of DZA and A17 were 45% and 47% of all the probe sets on the chip, respectively (Supplemental Table S1). An average of 34% of the probe sets on the chip produced signal intensity values with stem samples from the two alfalfa clones (Supplemental Table S1). In our previous work, an average of 46% and 54% of the *Medicago* probe sets on the chip produced present calls when hybridized with mRNA from *M. truncatula* first trifoliate and young roots, respectively [60]. A lower percentage, 41% and 44%, of detected probe sets was observed for developmentally comparable alfalfa first trifoliate and young roots, respectively [60]. These results are consistent with the fact that the majority (96%) of the target probe sets were designed from nucleotide sequence information of *M. truncatula* A17 [1]. Only 4% of the *Medicago* probe sets were based on nucleotide sequences from alfalfa cDNA libraries.

Hundreds of Differentially Expressed Probe Sets in Young and Old Stem Segments Show Similar Patterns of Expression in *M. truncatula* and Alfalfa

There was a very high degree of overlap in the sets of probe sets detected in young and old stem segments; approximately 98% of the total probe sets detected in each *Medicago* germplasm were expressed in both young and old stem segments (Supplemental Table S1). A *t* test ($p < 0.05$) in combination with the Benjamini and Hochberg false discovery rate [4] was employed for each *Medicago* germplasm to identify probe sets with significant transcript differences between young and old stem segments. Consistent with the total number of hybridizing probe sets, the numbers of probe sets significantly differentially expressed between young and old stem segments in both *M. truncatula* accessions were considerably higher than the numbers of probe sets significantly differentially expressed between young and old stem segments in the two alfalfa clones. Approximately 5,117 and 6,638 probe sets showed significantly different expression patterns between young and old stem segments of DZA and A17, respectively. Of the differentially expressed probe sets in A17 and DZA, 2,629 probe sets were in common between A17 and DZA (Fig. 2; Supplemental Table S2A). Of these, approximately 42% of differentially regulated probe sets showed increased transcript accumulation in old stem segments of A17 and DZA. A further 73 probe sets were uniquely expressed in old stem segments of both A17 and DZA, while 67 probe sets were uniquely expressed in young stem segments of both A17 and DZA.

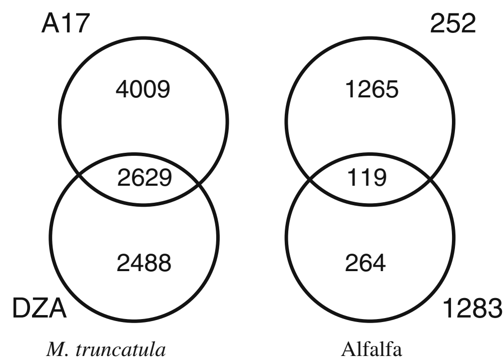

Fig. 2 Venn diagram summarizing the number of significantly different probe sets between young and old stem segments of *M. truncatula* and alfalfa. To correct for occurrence of false positives in the *t* test ($p<0.05$), multiple testing corrections were applied on the analysis [56] using the Benjamini and Hochberg false discovery rate [4]

For the alfalfa clones, approximately 1,384 and 383 probe sets were significantly differentially expressed between young and old stem segments of 252 and 1283, respectively. Of the differentially expressed probe sets in the alfalfa clones, 119 probe sets were in common to both 252 and 1283 (Fig. 2; Supplemental Table S2B). Approximately 13% of the differentially expressed probe sets showed significantly more transcript accumulation in old stem segments of the alfalfa clones. A further four probe sets were uniquely expressed in old stem segments, while 65 probe sets were uniquely expressed in young stem segments of both 252 and 1283. Overall, 52 probe sets were differentially expressed between stem segments across all four *Medicago* germplasms evaluated.

Functional Classification of Differentially Expressed Probe Sets and Visual Display Using MapMan Software

The differentially expressed probe sets between young and old stem segments of *M. truncatula* and alfalfa were assigned to functional categories as described in the "Methods". Functional classification of the 2,629 differentially expressed probe sets in young and old stem segments of the two *M. truncatula* accessions showed that numerous genes have predicted roles in transcriptional regulation and signal transduction (8%), primary and secondary metabolism (19%), as well protein modification and degradation (10%). A similar functional approach was used to classify the 119 differentially regulated probe sets in stem segments of alfalfa clones 252 and 1283. The largest putative functional categories included transcriptional regulation and signal transduction (9%), primary and secondary metabolism (26%), as well as enzyme families (14%) and transport function (8%).

To gain an overview of cellular and metabolic functional categories, the transcriptional profiles of the 2,629 differentially regulated probe sets in *M. truncatula* stem segments were visually displayed using MapMan software [61], as modified recently for the *Medicago* GeneChip [66]. The overview of cellular functions presented in Supplemental Figure S1 showed that probe sets with significantly higher transcript accumulation in young stem segments were largely categorized in DNA repair and synthesis, cell division, cell cycle and cell organization, hormone-related signaling, and enzyme family classes. On the other hand, many probe sets with significantly higher transcript abundance in old stem segments of *M. truncatula* have predicted function in regulation of transcription, hormone-related signaling, protein modification, and degradation (Supplemental Fig. S1).

An overview of differentially regulated probe sets representing metabolism and regulatory functions in *M. truncatula* is presented in Fig. 3a, b. Genes with higher transcript abundance in young stem segments include many implicated in lipid and flavonoid metabolism and cell wall degradation and modification, among other metabolic classes. In contrast, genes with significantly higher transcript abundance in old stem segments of *M. truncatula* were implicated in cellulose synthesis, regulation of transcription (transcription factors), signaling (receptor kinases and auxin-mediated signaling), protein modification, and protein degradation (Fig. 3a, b).

Genes Associated with Wall Modification Show Enhanced Expression in Young Stem Segments of *Medicago*

Cell wall-related genes that were differentially expressed between young and old stem segments were identified. Those genes with significantly higher transcript abundance in young stem segments of *M. truncatula* and alfalfa are presented in Tables 1 and 2, respectively. In young stem segments, most upregulated and/or preferentially expressed cell wall-related genes include expansins, beta-galactosidase, glycosyl hydrolase, xyloglucan endotransglucosylase/hydrolase (XET/XTH), proline-rich proteins, and fasciclin-like arabinogalactan proteins (AGP), among others. Many of the cell wall-related genes encode wall modifying proteins that play important roles in the relaxation of the rigid primary cell wall to allow elongation and extension during plant growth [11, 21, 22, 48]. We observed upregulated expression of two probe sets encoding XTHs, together with a uniquely expressed XET probe set (Mtr.45463.1.S1_at) in young stem segments of *M. truncatula*. There were also four expansin-like probe sets (Mtr.37590.1.S1_s_at, Mtr.9830.1.S1_at, Mtr.6653.1.S1_s_at, and Msa.1714.1.S1_at) that showed upregulated expression in young stem segments of *M.*

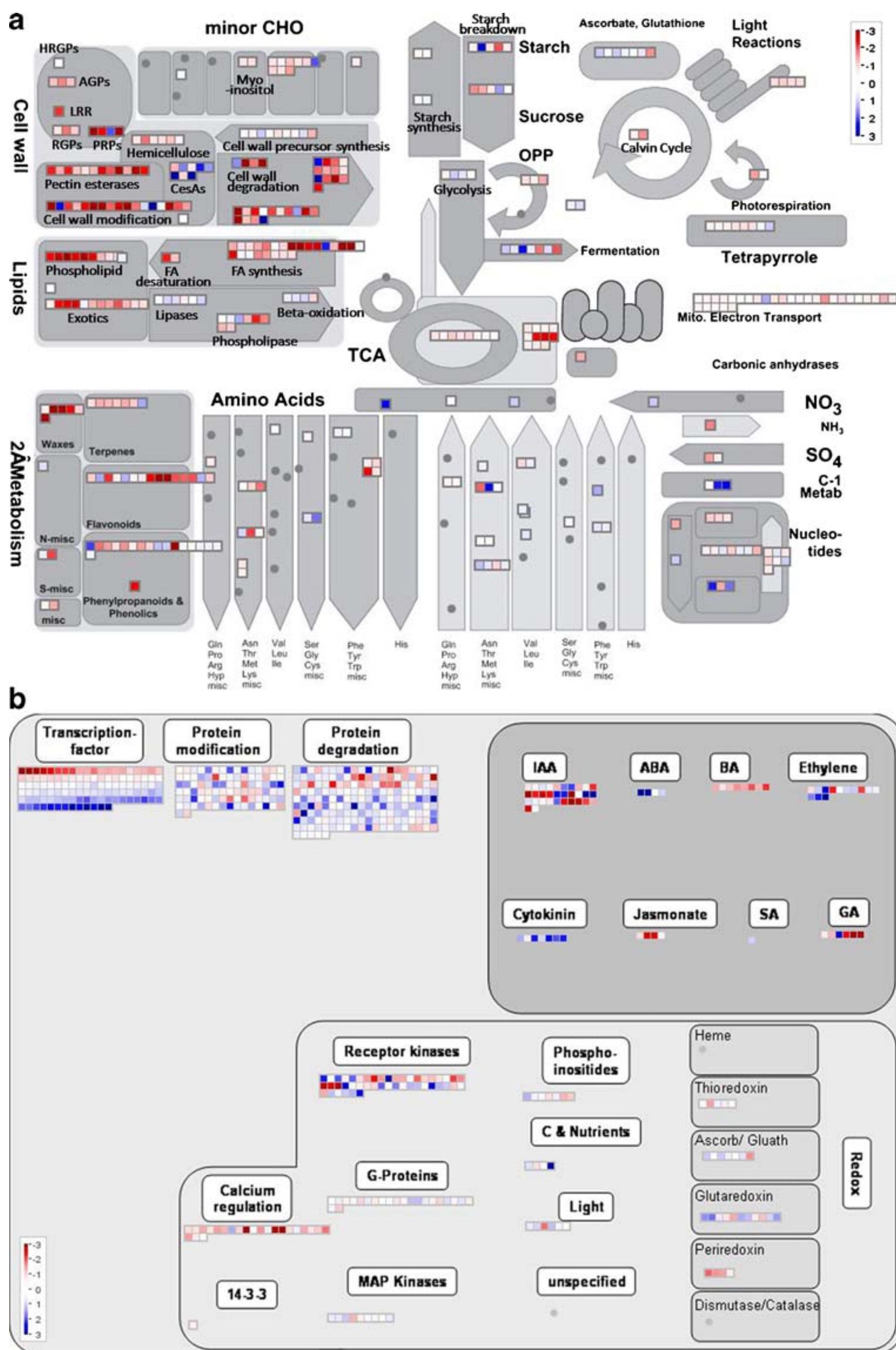

Fig. 3 Functional overview of significantly expressed probe sets in young versus old stem segments of *M. truncatula*. Figures depict MapMan software overview [61] for visualizing differential transcript abundance of probe sets with cellular functions associated with **a** primary and secondary metabolism and **b** regulatory and signal transduction. Expression ratios expressed as old/young stem internodes were $\log_2$ transformed and are shown as *red* and *blue squares* representing probe sets with upregulated expression in young and old stem segments, respectively

truncatula. Expansins are thought to be distributed primarily over the expanding cell wall and function as cell wall loosening proteins [48]. Our observations for the increased transcript accumulation of expansins, XTHs, and XET in young stem segments are consistent with the view that these cell wall proteins are expected to be predominantly active in primary walls of elongating tissues during plant growth [11, 21, 22, 48].

A number of probe sets that were upregulated in young stem segments of *M. truncatula* and alfalfa belong to genes

Table 1 Cell wall-related genes upregulated in young stem segments of *M. truncatula* A17 and DZA

Annotation	Probe set identifier	A17 ratio[a]	DZA ratio[a]	Number of probe sets[b]
Pathways of substrate generation				
4-Coumarate-CoA ligase-like	Mtr.44778.1.S1_at	0.32	0.21	
Cytochrome P450	Mtr.49936.1.S1_at	0.13	0.11	5 more probes
Polysaccharide synthesis				
Reversibly glycosylated protein	Mtr.27471.1.S1_s_at	0.31	0.27	1 more probe
Xyloglucan:xyloglucosyl transferase	Mtr.43130.1.S1_s_at	0.25	0.13	
Exostosin family protein	Mtr.1134.1.S1_s_at	0.35	0.25	12 more probes
Cell expansion and hemicellulose reassembly				
Expansin-like	Mtr.12712.1.S1_at	0.07	0.10	4 more probes
Endoxyloglucan transferase	Mtr.22601.1.S1_at	0.06	0.07	2 more probes
Xyloglucan endotransglucosylase hydrolase	Mtr.32541.1.S1_at	0.12	0.08	4 more probes
Endoxyloglucan transferase	Mtr.8521.1.S1_at	0.13	0.14	
Hydrolases				
Beta-1,3-glucanase-like protein	Mtr.43889.1.S1_at	0.25	0.26	4 more probes
Beta-galactosidase family	Mtr.51565.1.S1_s_at	0.14	0.12	4 more probes
Endo-1,4-beta-glucanase	Mtr.9073.1.S1_at	0.13	0.11	
Glucan endo-1,3-beta-glucosidase	Mtr.25573.1.S1_s_at	0.23	0.17	1 more probes
Glycosyl hydrolases/lyase family	Mtr.2639.1.S1_at	0.05	0.03	3 more probes
Polygalacturonase	Mtr.43598.1.S1_at	0.18	0.11	4 more probes
Pectate lyase	Mtr.1107.1.S1_at	0.10	0.07	
Esterases				
Pectinacetylesterase	Mtr.9861.1.S1_at	0.14	0.10	2 more probes
Pectin methylesterase	Msa.1350.1.S1_at	0.20	0.21	3 more probes
Pectinesterase	Mtr.37656.1.S1_at	0.15	0.11	3 more probes
Structural proteins				
Proline-rich protein	Mtr.38468.1.S1_at	0.21	0.15	2 more probes
Fasciclin-like arabiongalactan protein	Mtr.48645.1.S1_at	0.33	0.17	3 more probes
Arabinogalactan-protein	Mtr.11263.1.S1_at	0.41	0.31	
Differentiation and secondary wall formation				
Laccase	Mtr.13825.1.S1_at	0.22	0.14	
Peroxidase	Mtr.37542.1.S1_at	0.29	0.14	
Signaling and response mechanisms				
Phytocyanins: early nodulin-like	Mtr.13646.1.S1_at	0.05	0.04	
Leucine-rich repeat family protein	Mtr.48872.1.S1_at	0.28	0.18	4 more probes
Polygalacturonase inhibitor-like protein	Mtr.35738.1.S1_at	0.07	0.07	2 more probes
Receptor-like kinase	Mtr.31842.1.S1_at	0.26	0.17	3 more probes
Plastocyanin-like domain	Msa.2731.1.S1_at	0.33	0.48	1 more probe
Unknown GPI-anchored protein	Msa.2984.1.S1_at	0.14	0.17	8 more probes

[a] Ratios = signal intensity values of old stem segments over signal intensity values of young stem segments

[b] Number of probe sets indicates the number of additional probe sets with significant transcript abundance. Ratios are for the probe set with the highest expression ratio

encoding hydrolytic enzyme classes that appear to be involved in cell wall break down. Such genes include several homologs of glucanases, glucosyl hydrolases, and galactosidases, whose functions are largely related to cell wall expansion through hydrolysis of the pectin matrix (Tables 1 and 2).

Expression Patterns of Selected Marker Genes for Secondary Cell Wall Deposition Support Our Stem Segments Sampling Approach

Approximately 42% of the differentially expressed probes sets showed significantly upregulated expression in old

Table 2 Cell wall-related genes upregulated in young stem segments of alfalfa clones 252 and 1283

Annotation	Probe set identifier	252 ratio[a]	1283 ratio[a]	Number of probe sets[b]
Pathways of substrate generation				
UDP-glucose dehydrogenase	Msa.2895.1.S1_at	0.40	0.23	1 more probe
Polysaccharide synthesis				
Beta-6-xylosyltransferase	Mtr.11515.1.S1_at	0.38	0.29	
Endoxyloglucan transferase	Mtr.22601.1.S1_s_at	0.09	0.10	
Cell expansion and hemicellulose reassembly				
Expansin-like	Msa.1315.1.S1_at	0.08	0.07	2 more probes
Endoglucanase	Mtr.50574.1.S1_at	0.08	0.07	
Hydrolases/esterase				
Beta-galactosidase	Mtr.38644.1.S1_at	0.12	0.13	
Polygalacturonase-like	Mtr.43598.1.S1_at	0.19	0.19	
Endo-1,3(4)-beta-glucanase	Mtr.9073.1.S1_at	0.20	0.10	
Pectinesterase	Mtr.15938.1.S1_at	0.37	0.34	1 more probe
Structural proteins				
Proline-rich protein	Msa.1490.1.S1_at	0.10	0.07	4 more probes
Arabinogalactan-proteins	Mtr.11263.1.S1_at	0.25	0.26	
Fasciclin-like AGPs 1	Mtr.5307.1.S1_s_at	0.23	0.25	
Signaling and response mechanisms				
Receptor-like kinase	Msa.1188.1.S1_at	0.03	0.03	1 more probe
Phytocyanins: early nodulin-like	Mtr.19874.1.S1_at	0.40	0.43	
Copper ion binding/oxidoreductase	Mtr.15975.1.S1_s_at	0.06	0.10	1 more probe
Protease inhibitor	Mtr.20794.1.S1_at	0.25	0.23	
Pectinesterase inhibitor	Mtr.18676.1.S1_at	0.39	0.46	

[a] Ratios = signal intensity values of old stem segments over signal intensity values of young stem segments

[b] Number of probe sets indicates the number of additional probe sets with significant transcript abundance. Ratios are for the probe set with the highest expression ratio

stem segments of *M. truncatula*. Many genes that were upregulated in old stem segments showed similarities with proteins associated with secondary cell walls including cellulose synthases (*CESA*), COBRA-like protein 4 precursor (*COBL4*), never in mitosis gene A (NIMA)-related protein kinase, peroxidise, 4-coumarate:CoA ligase, cytochrome P450, cinnamyl alchol dehydrogenase, and some fasciclin-like AGPs (Tables 3 and 4). *COBL4*, some members of the *CESA* family, as well as selected lignin biosynthesis genes, are known marker genes for secondary wall biosynthesis in plants [6, 7, 46]. Our transcript profiling results coincide well with upregulated expression patterns of such secondary cell wall marker genes in old stem segments and appears to provide strong support for our stem segment sampling approach for studying cell wall genomics in *Medicago*. In most previously reported studies using different stem segments, transcript abundance of secondary cell wall marker genes including those involved in lignification was the highest in older stem segments and showed a decreased pattern of expression in stem segments near the apical meristem [7, 17, 49].

In *Arabidopsis*, the extracellular glycosylphosphatidyl inositol (GPI)-anchored protein COBL4 appears to participate in cell expansion and was required for cellulose biosynthesis in secondary walls [6, 7, 52, 53]. Mutation of the *Arabidopsis COBL4* gene (At5g15630) by T-DNA insertion resulted in plants showing a moderate irregular xylem (*irx6*) phenotype with significantly reduced levels of cellulose stem strength that resulted in mutant plants with easily broken stems [7]. A rice mutant described as *brittle culm1* (*bc1*) was found to be a functional ortholog of the AtCOBL4 gene, as mutations in the rice gene resulted in reduced cell wall thickness affecting the mechanical strength of rice plants [40]. Here, a *Medicago* homolog of the COBL4 gene (Mtr.5947.1.S1_at) showed more than 23- and 50-fold higher transcript abundance in old stem segments of DZA and A17 compared to young stem segments, respectively (Table 3). This probe set was also upregulated in old stem segments of both alfalfa clones.

The *Arabidopsis* genome contains a superfamily of approximately 41 predicted *CESA*-like genes [30, 50], and the differential expression pattern of *CESA* genes in primary

Table 3 Top 30 upregulated cell wall-related genes in old stem segments of *M. truncatula* A17 and DZA

Annotation	Probe set identifier	A17 ratio[a]	DZA ratio[a]	Number of probe sets[b]
Pathways of substrate generation				
Cytochrome P450	Mtr.6728.1.S1_at	5.8	4.1	2 more probes
NAD-dependent epimerase/dehydratase family protein	Mtr.37829.1.S1_at	2.3	1.6	
UDP-glucose 4-epimerase	Msa.2601.1.S1_at	2.0	2.2	
Trans-cinnamate 4-monooxygenase	Msa.2979.1.S1_at	2.2	2.4	
Cinnamyl alcohol dehydrogenase	Mtr.49106.1.S1_at	1.4	1.5	1 more probe
Polysaccharide synthesis				
Cellulose synthase-like	Mtr.6551.1.S1_at	3.8	2.8	
Cellulose synthase	Mtr.21515.1.S1_at	5.7	4.2	
Cellulose synthase, catalytic subunit	Mtr.33547.1.S1_at	3.2	2.5	
Cellulose synthase	Mtr.5123.1.S1_at	29.9	13.3	
Exostosin family protein	Mtr.12707.1.S1_at	2.7	3.0	
Glucosyltransferase family protein	Mtr.17803.1.S1_at	4.8	2.5	3 more probes
Cell expansion and hemicellulose reassembly				
Xyloglucan endotransglucosylase hydrolase	Msa.1770.1.S1_at	4.2	7.0	
Hydrolases/esterase				
Beta-galactosidase	Mtr.43150.1.S1_at	28.7	17.9	1 more probe
Glycosyl hydrolase family protein	Mtr.2372.1.S1_at	3.3	1.9	
Polygalacturonase-like protein	Mtr.43324.1.S1_s_at	3.0	2.5	
Glucan endo-1,3-beta-glucosidase	Mtr.8969.1.S1_at	1.4	1.7	
Pectinesterase family protein	Mtr.40223.1.S1_at	5.3	10.8	
Structural proteins				
Hydroxyproline-rich glycoprotein	Mtr.43240.1.S1_at	1.1	1.2	
Differentiation and secondary wall formation				
Peroxidase	Msa.1639.1.S1_at	7.0	5.0	1 more probe
Signaling and response mechanisms				
NIMA-related protein kinase	Mtr.9213.1.S1_at	4.5	4.4	
Leucine-rich repeat transmembrane protein kinase	Mtr.449.1.S1_at	3.5	3.8	2 more probes
COBL4/IRX6 (COBRA-LIKE4)	Mtr.5947.1.S1_at	56.5	23.2	
Receptor-like kinases	Mtr.18579.1.S1_at	8.0	3.4	12 more probes
Protein kinase	Mtr.24954.1.S1_s_at	2.4	2.7	
Pathogenesis-related protein	Mtr.9418.1.S1_s_at	2.3	2.3	
Ser/Thr protein kinase	Mtr.30764.1.S1_at	1.9	2.7	4 more probes
Thaumatin, pathogenesis-related	Mtr.17914.1.S1_at	1.9	1.3	1 more probe
S-receptor kinase-like protein	Mtr.34147.1.S1_at	1.7	1.8	
Unknown GPI-anchored protein	Mtr.38207.1.S1_at	2.4	2.8	2 more probes
Peptidase	Mtr.16993.1.S1_at	2.2	2.4	1 more probe
Aspartyl protease-like	Mtr.27603.1.S1_at	1.4	1.6	

[a] Ratios = signal intensity values of old stem segments over signal intensity values of young stem segments

[b] Number of probe sets indicates the number of additional probe sets with significant transcript abundance. Ratios are for the probe set with the highest expression ratio

versus secondary cell walls is well documented. Based on genetic experiments and gene-expression analyses, three *Arabidopsis CESA* genes (AtCesA1, AtCesA3, and AtCesA6) typify primary walls and are coexpressed during primary cell wall formation [47], while three other *CESA* genes (AtCesA4/*IRX*5, AtCeSA7/*IRX*3, and AtCeSA8/*IRX*1) are involved in cellulose biosynthesis in secondary cell walls [47, 58, 59, 62]. In *Arabidopsis*, mutations of certain *CESA* genes resulted in the collapse of the secondary cell wall of xylem (irregular xylem), indicating that *CESA* genes are required for biosynthesis of secondary cell walls [62]. The *Medicago* chip contains approximately 28 probe sets encoding *CESA* genes. One *CESA* probe set (Mtr.33499.1.S1_at) was uniquely expressed in old stem segments of both

Table 4 Cell wall-related genes upregulated in old stem segments of alfalfa clones 252 or 1283

Annotation	Probe set identifier	252 ratio[b]	1283 ratio[b]
Pathways of substrate generation			
Cytochrome P450	Mtr.20710.1.S1_at	2.9	3.8
Cinnamyl alcohol dehydrogenase	Mtr.49106.1.S1_s_at[a]	1.2	1.4
N-Hydroxycinnamoyl benzoyltransferase	Mtr.34427.1.S1_at	1.4	0.5
Polysaccharide synthesis			
Cellulose synthase	Mtr.5123.1.S1_at	3.0	2.8
Cellulose synthase	Mtr.44557.1.S1_at	3.6	3.8
Cellulose synthase	Mtr.28768.1.S1_at	3.7	8.6
Hydrolases/esterase			
Beta-galactosidase	Mtr.43150.1.S1_at	2.4	6.0
Glycoside hydrolase	Mtr.50907.1.S1_at[a]	1.4	1.9
Pectinacetylesterase	Mtr.40699.1.S1_at	2.8	4.9
Structural proteins			
Hydroxyproline-rich glycoprotein-like	Mtr.43240.1.S1_at	1.2	1.0
Beta-Ig-H3 fasciclin	Mtr.50900.1.S1_at	3.0	6.9
Beta-Ig-H3 fasciclin	Mtr.17361.1.S1_at	2.7	5.5
Beta-Ig-H3 fasciclin	Mtr.18563.1.S1_at	3.0	7.1
Differentiation and secondary wall formation			
Peroxidase	Mtr.12601.1.S1_at	2.7	5.4
Signaling and response mechanisms			
Mitogen-activated protein kinase 4	Mtr.39475.1.S1_at[a]	1.5	1.6
Protein kinase	Mtr.12713.1.S1_at	1.3	1.1
Receptor protein kinase	Mtr.6363.1.S1_at	2.1	1.4
Receptor protein kinase	Mtr.44364.1.S1_at	2.3	2.2
Receptor protein kinase	Mtr.38398.1.S1_at	2.4	1.3
Receptor protein kinase	Mtr.4995.1.S1_at	1.3	1.6
Serine–threonine protein kinase	Mtr.44992.1.S1_s_at[a]	1.6	1.6

[a] Probe set identifiers are differentially regulated in young versus old stem internodes of alfalfa clone 1283. The rest of the probe sets are differentially regulated in young versus old stem internodes of alfalfa clone 252

[b] Ratios = signal intensity values of old stem segments over signal intensity values of young stem segments

A17 and DZA, while 15 other *CESA* probe sets showed particularly high transcript abundance in old stem segments of A17 and DZA (Fig. 4). The remaining *Medicago CESA* probes showed increased transcript abundance in young stem segments.

Differentially Expressed Transcription Factors and Signal Transduction Genes Suggest Transcriptional Control of Stem Development and Growth in *Medicago*

Transcription factors are key global regulators of gene expression and are known to play critical roles in many biological processes, including the regulation of cell wall development in plants. In *Arabidopsis*, TF-encoding genes make up approximately 6% (about 1,800) of the total number of genes including about 72 WRKY family genes, more than 600 zinc finger proteins, and 199 MYB and MYB-related transcription factors [19, 27, 51, 57]. Sequencing of the *M. truncatula* genome is in progress. Using BLAST analysis of the available *M. truncatula* genome sequencing data, Udvardi et al. [63] identified about 1,084 TF genes. We found that the Affymetrix *Medicago* chip contains approximately 1,870 probe sets that by amino acid homology could be classified as putative TFs by established criteria (http://datf.cbi.pku.edu.cn/). Approximately 113 putative TF probe sets were significantly differentially expressed in young versus old stem segments of both A17 and DZA; approximately 65% of these TFs showed increased transcript abundance in old stem segments of *M. truncatula*. The differentially expressed TF probe sets in *M. truncatula* stems represented 35 TF families (Supplemental Table S3). In alfalfa, approximately five putative TF probe sets were differentially expressed in young versus old stem segments of both 252 and 1283. Differentially regulated TF probe sets in alfalfa stems represented bHLH (Mtr.20533.1.S1_at and Mtr.33785.1.S1_at), CAMTA (Mtr.42126.1.S1_at), and APETALA2/ethylene-responsive

Fig. 4 Cellulose synthases show differential expression patterns in stem segments of *M. truncatula*. The heat map shows ratio of signal intensity values in old stem segments of each *Medicago* ecotype relative to signal intensity values in young stem segments of the same ecotype. *Red* indicates upregulated expression, *green* indicates downregulation, and *yellow* indicates no change in expression profiles compared to young internodes

element binding protein family (AP2/EREBP; Mtr.2744.1.S1_at and Mtr.41294.1.S1_at) TF families.

The list of differentially expressed TF families in *M. truncatula* stem segments consists of several plant-specific TF families including AP2-EREBP, auxin/indole-3-acetic acid (Aux/IAA), auxin-responsive factor (ARF), GRAS, NAC, and WRKY families. The precise contribution of the differentially regulated TFs in modulating cell wall biosynthesis in *Medicago* stems remains to be determined, although some or all of the differentially expressed TFs may have important roles in other plant developmental processes within *Medicago* stems. Nevertheless, our transcript profiling results were consistent with the lists of putative wall-associated TFs identified by transcript profiling in several plant species [reviewed by 13, 69]. For instance, the Aux/IAA genes are plant-specific TF gene families that participate in auxin-regulated transcriptional control of gene expression [38, 45]. Wall synthesis during plant development and growth was shown to be influenced by endogenous levels of hormones, and additional modifications can be induced by biotic or abiotic stresses [32]. Auxin triggers a specific signal transduction pathway that influences apical dominance, vascular tissue development, cell elongation, and tissue patterning. Expression of Aux/IAA genes is auxin inducible, which is expected to provide a negative-feedback loop for auxin responses by forming homo- and hetrodimers with Aux/IAAs or other TFs such as ARF proteins. There were several ARF probe sets that were differentially regulated in *Medicago* stem segments.

With regard to signal transduction, at least 4% of the differentially regulated probe sets in young and old stem segments of *M. truncatula* and alfalfa germplasm encode genes that are implicated in signal transduction cascades. Many differentially regulated signaling genes include homologs of receptor-like protein kinases (RLKs), several GPI-anchored proteins of unknown function, many Ser/Thr protein kinase/phosphatases, and genes with interacting domains such as leucine-rich repeat (LRR) containing protein kinases, enzyme inhibitors, and proteases. RLKs and LRR domain containing RLKs have been implicated in many plant developmental processes including controlling fiber development in cotton [41].

Identification of Additional Candidate Genes for Secondary Cell Walls Through Analysis of Coexpressed Probe Sets with Selected Marker Genes

To further identify candidate genes that may have roles associated with secondary cell wall deposition in *Medicago*, we extended our analysis to include a large collection of publicly available *M. truncatula* microarray data for analysis of coexpression patterns using marker genes. Recently, similar coexpression approaches were successfully used in *Arabidopsis* to identify genes required for cellulose synthesis in primary or secondary cell walls [7, 46]. Marker genes associated with secondary cell wall biosynthesis in *Arabidopsis* used for the coexpression analysis included three *CESA* genes (AtCesA4/*IRX*5, AtCeSA7/*IRX*3, and AtCeSA8/*IRX*1) and COBL4 [6, 46, 59]. We used the expression profiles of a *CESA* gene (represented by Mtr.5123.1.S1_at), as well as a *COBL4* homolog (represented by Mtr.5947.1.S1_at), as reference probe sets to identify coregulated genes in the publicly available *M. truncatula* microarray data. These two probe sets were selected as reference points for our analysis because (a) the deduced protein sequence of Mtr.5123.1.S1_at was the probe set that showed the greatest similarity (approximately 80% amino acid sequence identity) to AtCesA8, which is known to be required for cellulose synthesis during secondary cell wall formation [58]; (b) the deduced protein sequence of Mtr.5947.1.S1_at was the probe set with the greatest similarity (approximately 76% amino acid sequence identity) to that of AtCOBL4, which in prior studies was found to be required for secondary cell wall synthesis and was among the genes that showed a high level of coexpression with secondary cell wall-associated *CESA* genes [7, 46]; and (c) these two probe sets were among those with highest expression ratios in old stem segments of *Medicago*.

Our analysis revealed 213 probe sets that were coexpressed ($R^2 \geq 0.7$) with *COBL4* and/or *CESA* probe sets (Supplemental Table S4), including those with putative functions associated with cellulose synthesis, cell wall structural components including fasciclin-like AGPs, laccases, peroxidases, and putative signaling and regulators such as no apical meristem (NAM)-like (NAC-like), WRKY, and MYB family transcription factors as well as several receptor like protein kinases (Table 5). Although the coexpressed probe sets implicated in signal response and TF genes have not been investigated experimentally, many of the coexpressed genes suggest novel finds among secondary cell wall-associated genes in *Medicago*. Our list of coexpressed genes included those that were previously reported to be secondary cell wall coregulated genes [7, 46]. For example, several *Medicago* fasciclins (MtFLA) were coexpressed with both marker *CESA* and *COBL4* probe sets (Table 5). In previous studies, two *Arabidopsis* genes, AtFLA11 (At5g03170) and AtFLA12 (At5g60490), were among those that were most highly coexpressed with *AtCesA* genes required for secondary cell wall formation [7, 46]. *Arabidopsis* contains at least 21 fasciclin-like genes [36], and a mutation in the fasciclin domain of one of the fasciclin-like genes (AtFLA4) resulted in aberrant cell expansion in *Arabidopsis* [55]. Plant fasciclins are a subgroup of AGPs that contain unique fasciclin domains that also occur in proteins from bacteria, mammals, sea urchins, and yeast and are thought to be involved in cell adhesion [16, 36]. Fasciclin-like AGPs were highly abundant in *Populus* spp. with strong preferential expression in xylem cells during formation of cellulose-rich tension wood [2, 39]. Nevertheless, many poplar fasciclins appear to lack orthologs in the *Arabidopsis* genome [2, 39].

The *Medicago* chip contains approximately 17 probe sets encoding fasciclin-like genes (MtFLAs). Of these, 11 probe sets were designed from sequences based on gene predictions of the *M. truncatula* genome sequencing project. The coding sequence of MtFLAs, for which the complete sequence information is available, ranges in size from 931 to 1,971 nucleotides. Our analysis showed that many of the MtFLAs appear to contain several shared features: (a) an N-terminal signal peptide sequence, (ii) 5′ and 3′ untranslated regions, (c) a fasciclin domain, and (d) absence of introns. The exception was a gene represented by probe set Mtr.48645.1.S1_at that displayed two fasciclin domains along the coding sequence and contained a single intron of 673 nucleotides. Proteins encoded by some of the MtFLAs also contained one or two transmembrane domains at their N- and/or C-terminal regions.

In an attempt to construct a MtFLA expression atlas, the tissue-specific and developmental regulated expression patterns of all 17 MtFLAs on the chip were evaluated using the publicly available *Medicago* microarray data [3, 31] and data from this study. Three expression clusters were evident (Fig. 5). The first expression cluster comprised nine MtFLA probes sets that showed particularly high transcript abundance (more than tenfold difference) in old stem segments compared to young stem segments. Interestingly, these probe sets showed highly coregulated expression patterns with *COBL4* and *CESA* genes (Table 5) and also showed very strong gene expression in petioles, stems, and young roots of 4-week-old A17 plants (Fig. 5). Very strong gene expression by members of this clade was also observed in young nodules of A17 roots at 4 days postinoculation (pi) with *Sinorhizobium meliloti*, presumably because the young nodule samples also contained some root tissues attached to the nodules. However, very low expression was observed in nodule samples at 10 and 14 days pi, as well as in mature nodules. The second expression cluster comprised three MtFLA probe sets

Table 5 List of most highly coexpressed probe sets for *COBL4* and *CESA* genes using publicly available *M. truncatula* A17 microarray data

Annotation	Probe set identifier	*COBL4* R^2	*CESA* R^2
Pathways of substrate generation			
Cytochrome P450	Mtr.25160.1.S1_at	0.703	0.724
Cytochrome P450	Mtr.20710.1.S1_at	0.906	0.757
Cytochrome P450	Mtr.6322.1.S1_s_at	0.620	0.720
Ferulate-5-hydroxylase precursor	Mtr.42553.1.S1_s_at	0.910	0.880
UDP-glucose 4-epimerase	Mtr.28802.1.S1_at	0.890	0.874
UDP-glucose 4-epimerase	Mtr.39188.1.S1_at	0.703	0.722
Polysaccharide synthesis			
Cellulose synthase	Mtr.28768.1.S1_at	0.990	0.913
Cellulose synthase	Mtr.33499.1.S1_at	0.976	0.849
Cellulose synthase, catalytic subunit	Mtr.44557.1.S1_at	0.930	0.980
Cellulose synthase	Mtr.5123.1.S1_at	0.898	1.000
Glycosyl transferase family 8	Mtr.41311.1.S1_at	0.597	0.732
Glycosyl transferase family 8	Mtr.44353.1.S1_s_at	0.694	0.788
Glycosyl transferase family 8	Mtr.9912.1.S1_at	0.538	0.737
Cell expansion and hemicellulose reassembly			
Expansin-related protein 1 precursor	Mtr.35168.1.S1_s_at	0.738	0.721
Expansin-related protein 1 precursor	Mtr.41777.1.S1_at	0.747	0.702
Beta-galactosidase	Mtr.43150.1.S1_at	0.912	0.870
Membrane-anchored endo-1,4-beta-glucanase	Mtr.26604.1.S1_at	0.638	0.773
Structural proteins			
Arabinogalactan protein	Mtr.24717.1.S1_s_at	0.925	0.885
Arabinogalactan protein-like	Mtr.10992.1.S1_at	0.853	0.839
Beta-Ig-H3 fasciclin	Mtr.17361.1.S1_at	0.937	0.893
Beta-Ig-H3 fasciclin	Mtr.18380.1.S1_at	0.909	0.886
Beta-Ig-H3 fasciclin	Mtr.18563.1.S1_at	0.927	0.955
Beta-Ig-H3 fasciclin	Mtr.50897.1.S1_at	0.978	0.936
Beta-Ig-H3 fasciclin	Mtr.50900.1.S1_at	0.971	0.909
Beta-Ig-H3 fasciclin	Mtr.51607.1.S1_at	0.957	0.857
Beta-Ig-H3 fasciclin	Mtr.17362.1.S1_at	0.673	0.728
Fasciclin-like AGP 10	Mtr.13136.1.S1_at	0.962	0.908
Fasciclin-like AGP 10	Mtr.13136.1.S1_s_at	0.974	0.934
Fasciclin-like AGP 9	Mtr.45260.1.S1_at	0.927	0.929
Differentiation and secondary wall formation			
Laccase	Mtr.42734.1.S1_at	0.721	0.700
Laccase	Mtr.13653.1.S1_at	0.630	0.709
Laccase	Mtr.39737.1.S1_at	0.557	0.722
Peroxidase	Mtr.7078.1.S1_at	0.616	0.740
Peroxidase	Mtr.4733.1.S1_at	0.747	0.726
Peroxidase	Mtr.4733.1.S1_s_at	0.745	0.756
Peroxidase	Mtr.51876.1.S1_at	0.764	0.813
Signaling and response mechanisms			
COBL4/IRX6 (COBRA-LIKE4)	Mtr.5947.1.S1_at	1.000	0.898
Extensin-like protein	Mtr.32593.1.S1_at	0.732	0.852
Leucine-rich receptor-like protein kinase	Mtr.13683.1.S1_at	0.721	0.709
Leucine-rich receptor-like protein kinase 1	Mtr.12186.1.S1_at	0.564	0.744
LysM domain-containing receptor-like kinase 7-related	Mtr.51429.1.S1_s_at	0.763	0.779
Peptidase	Mtr.16048.1.S1_at	0.544	0.741

Table 5 (continued)

Annotation	Probe set identifier	*COBL4* R^2	*CESA* R^2
Protein kinase-like	Mtr.16216.1.S1_at	0.699	0.718
Protein kinase-like	Mtr.21071.1.S1_at	0.647	0.745
Protein-serine–threonine kinase	Mtr.32154.1.S1_at	0.719	0.775
Receptor kinase-like protein	Mtr.32077.1.S1_at	0.662	0.798
Receptor protein kinase	Mtr.38398.1.S1_at	0.657	0.725
Receptor protein-like	Mtr.11833.1.S1_at	0.803	0.816
Receptor-kinase like protein	Mtr.38555.1.S1_at	0.752	0.707
Receptor-like	Mtr.2463.1.S1_s_at	0.626	0.706
Receptor-like kinases	Mtr.5261.1.S1_at	0.573	0.745
Receptor-like protein kinase	Mtr.2022.1.S1_at	0.803	0.797
Serine–threonine kinase receptor-like protein	Mtr.13729.1.S1_at	0.574	0.745
Toll-like receptor 6 precursor	Mtr.39114.1.S1_at	0.618	0.741
Transcription factors			
APETALA2/ethylene-responsive element binding protein family	Mtr.9223.1.S1_at	0.796	0.721
Auxin-responsive factor AUX/IAA (ARF19)	Mtr.39550.1.S1_at	0.624	0.791
ARF4-ARF	Mtr.26216.1.S1_at	0.574	0.770
ARF4-ARF	Mtr.26217.1.S1_at	0.658	0.725
ARR1—two-component responsive regulator family protein (GARP-ARR-B)	Mtr.42943.1.S1_at	0.540	0.701
bHLH family protein	Mtr.13973.1.S1_s_at	0.511	0.711
Brassinosteroid signaling positive regulator-related (BES1)	Mtr.5565.1.S1_at	0.681	0.723
Homeobox-leucine zipper family (HB)	Mtr.7137.1.S1_at	0.586	0.712
Homeobox-leucine zipper family (HB)	Mtr.23994.1.S1_at	0.667	0.717
MYB family transcription factor (MYB103)	Mtr.34412.1.S1_at	0.741	0.852
MYB family transcription factor (MYB115)	Mtr.41037.1.S1_at	0.725	0.742
NAM family (NAC)	Mtr.5547.1.S1_s_at	0.666	0.751
NAM family (NAC)	Mtr.39376.1.S1_at	0.660	0.736
NAM family (NAC)	Mtr.27801.1.S1_at	0.685	0.773
NAM family n (NAC)	Mtr.1813.1.S1_at	0.753	0.792
NAM family (NAC)	Mtr.3002.1.S1_at	0.634	0.736
Short-root transcription factor (GRAS)	Mtr.35871.1.S1_at	0.717	0.816
Short-root transcription factor (GRAS)	Mtr.7886.1.S1_at	0.818	0.853
WRKY family transcription factor	Mtr.51555.1.S1_at	0.603	0.722
WRKY13 family transcription factor	Mtr.45854.1.S1_at	0.587	0.732
WRKY20 family transcription factor	Mtr.9902.1.S1_s_at	0.533	0.792
WRKY20 family transcription factor	Mtr.35329.1.S1_s_at	0.516	0.723

ARF auxin-responsive factor, *NAM* no apical meristem

(Mtr.17362.1.S1_at, Mtr.50904.S1_at, Mtr.48994.S1_at) that showed 1.5- to twofold more transcript abundance in old stem segments compared to young stem segments. These probes also showed enhanced gene expression in petioles and stems of 4-week-old A17 plants. The third expression cluster consisted of those MtFLAs (Mtr.17392.1.S1_at, Mtr.37610.1.S1_s_at, Mtr.42768.1.S1_at, Mtr.42950.1.S1_at, and Mtr.48645.1.S1_at) with considerably down-regulated expression in old stem segments, indicating enhanced transcript abundance predominantly in young stem segments of A17. Members of this expression cluster also showed downregulated expression in roots, petioles, and stems of 4-week-old A17 plants. These results are consistent with previous genetic analysis and transcript profiling studies that suggested a role for fasciclin-like AGPs in both primary and secondary wall formation [39, 46, 55]. There is limited information in the functional importance of fasciclins in plants.

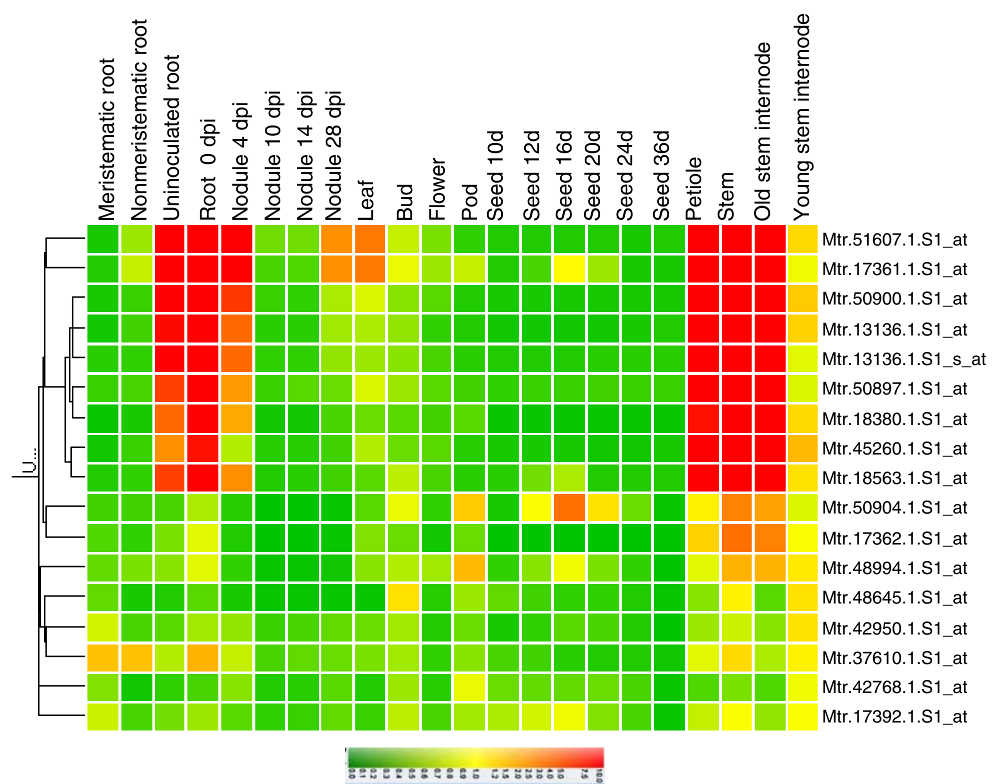

Fig. 5 Transcript atlas of *Medicago* fasciclins (MtFLAs). Results include publicly available *M. truncatula* A17 microarray data as described in methods. The heat map represents ratio of gene expression in each organ relative to signal intensity values in young stem segments of A17. *Red* indicates upregulated expression, *green* indicates downregulation, and *yellow* indicates no change in expression profiles compared to young internodes

The phylogenetic position of MtFLAs was compared with FLAs homologs in the poplar and *Arabidopsis* genomes. We performed multiple sequence alignments of deduced amino acid sequences of 11 MtFLAs for which a complete coding sequence is available, along with the amino acid sequences of *Arabidopsis* and poplar fasciclins. Amino acid sequences of known classical AGPs were included for comparison in phylogenetic tree construction. In the phylogenetic tree, classical AGPs formed a tight cluster that was distinct from FLAs, while FLAs were clustered in about five subclades (Supplemental Figure S2). In two subclades, which contained one or no *Arabidopsis* FLAs, numerous MtFLAs were clustered very closely with poplar fasciclins (Supplemental Figure S2).

Conclusions

As an experimental system, the genus *Medicago* has the advantages of a model plant with ample genetic and genomic resources (*M. truncatula*) and a cultivated crop (*M. sativa*) with its economic importance as a forage plant, ability to improve soil fertility status, and potential to be a bioenergy feedstock. The large number of differentially regulated cell wall-related genes in *M. truncatula* will be valuable for exploring genetic systems controlling primary and secondary cell wall deposition in plants and may facilitate gene discovery and improvement of biomass production of quality traits in cultivated alfalfa or related dicots.

The phylogenetic grouping of the Eudicots shows that the eurosid I clade, in addition to Fabales (legumes), contains the Malpighiales (where polar and willow are placed), among many other woody species [25]. By contrast, *Arabidopsis* in the Brassicales lineage is found in a different branch of plant species with the Rosids, the eurosid II clade. Beyond legumes, *M. truncatula* may have utility as a genetic model for cell wall development in closely related woody dicots and offers a new approach to study an expanded repertoire of agronomic traits of value in other crops. Multiple model plants and a systems approach will be needed to decipher the regulation of cell wall biogenesis in plants. It is probable that *M. truncatula* could help to fill in the knowledge gap concerning divergent genes that cannot be addressed in *Arabidopsis*. Moreover, *M. truncatula*, a short-lived annual, will facilitate experimental analysis of gene function that would be difficult in woody dicots including poplar, due to their long generation times.

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