MAIZEWALL. Database and Developmental Gene Expression Profiling of Cell Wall Biosynthesis and Assembly in Maize [1][W]

Sabine Guillaumie, Hélène San-Clemente, Caroline Deswarte, Yves Martinez, Catherine Lapierre, Alain Murigneux, Yves Barrière, Magalie Pichon, and Deborah Goffner*

Université Paul Sabatier, Centre National de la Recherche Scientifique, Unité Mixte de Recherche 5546, 31326 Castanet-Tolosan, France (S.G., H.S.-C., C.D., Y.M., M.P., D.G.); Institut National de la Recherche Agronomique (INRA), Unité de Génétique et d'Amélioration des Plantes Fourragères, 86600 Lusignan, France (S.G., Y.B.); INRA-Institut National Agronomique Paris-Grignon, Unité de Chimie Biologique, 78850 Thiverval-Grignon, France (C.L.); and Biogemma, Campus Universitaire des Cézeaux, 63170 Aubière, France (A.M.)

An extensive search for maize (*Zea mays*) genes involved in cell wall biosynthesis and assembly has been performed and 735 sequences have been centralized in a database, MAIZEWALL (http://www.polebio.scsv.ups-tlse.fr/MAIZEWALL). MAIZEWALL contains a bioinformatic analysis for each entry and gene expression data that are accessible via a user-friendly interface. A maize cell wall macroarray composed of a gene-specific tag for each entry was also constructed to monitor global cell wall-related gene expression in different organs and during internode development. By using this macroarray, we identified sets of genes that exhibit organ and internode-stage preferential expression profiles. These data provide a comprehensive fingerprint of cell wall-related gene expression throughout the maize plant. Moreover, an in-depth examination of genes involved in lignin biosynthesis coupled to biochemical and cytological data from different organs and stages of internode development has also been undertaken. These results allow us to trace spatially and developmentally regulated, putative preferential routes of monolignol biosynthesis involving specific gene family members and suggest that, although all of the gene families of the currently accepted monolignol biosynthetic pathway are conserved in maize, there are subtle differences in family size and a high degree of complexity in spatial expression patterns. These differences are in keeping with the diversity of lignified cell types throughout the maize plant.

Cell walls play an essential role in determining cell size and shape and, as a result, contribute to functional specialization of different cell types. The physicochemical nature of the cell wall is highly dynamic, changing dramatically during cell growth and expansion. During normal growth and development, an expanding cell must have a cell wall that is flexible enough to allow for rapid increase in cell volume, whereas, when expansion ceases, the wall must afford rigidity. During the differentiation of specialized cell types, such as fibers and xylem tracheary elements (TEs), in addition to primary walls, secondary walls are subsequently deposited to ensure additional mechanical strength and solute conduction. Beyond normal cell wall dynamics, during growth and development, wall structure may also change as a function of biotic or abiotic stress. This may occur through a wide variety of mechanisms, including lignin (Mitchell et al., 1999) or callose (Rodriguez-Galvez and Mendgen, 1995) deposition, secretion of structural proteins (Bestwick et al., 1995), and oxidative cross-linking of existing Pro-rich or Hyp-rich glycoproteins (Bradley et al., 1992; Otte and Barz, 2000).

As a first step in obtaining information about individual cell wall-related gene products and their role in plant growth and development, many genomic approaches have been undertaken. An extensive genomic cell wall database has recently become available in Arabidopsis (*Arabidopsis thaliana*; Girke et al., 2004). In this Cell Wall Navigator database, more than 5,000 putative cell wall genes coding for enzymes and structural proteins known to be involved in primary wall metabolism have been assembled. It has been predicted that over 100 protein families with over 1,000 members are involved in cell wall-related processes (Henrissat et al., 2001). As for genomic initiatives of secondary walls, large expressed sequence tag (EST) and microarray datasets have been generated for agronomic and model species, including cotton (*Gossypium hirsutum*) fibers (Arpat et al., 2004), poplar (*Populus tremuloides*; Hertzberg et al., 2001) and pine

[1] This work was supported by the Génoplante Programme, Institut National de la Recherche Agronomique, and the Centre National de la Recherche Scientifique.

* Corresponding author; e-mail goffner@scsv.ups-tlse.fr; fax 33–562–19–35–02.

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantphysiol.org) is: Deborah Goffner (goffner@scsv.ups-tlse.fr).

[W] The online version of this article contains Web-only data.
www.plantphysiol.org/cgi/doi/10.1104/pp.106.086405

(*Pinus sylvestris*; Pavy et al., 2005) wood, zinnia (*Zinnia elegans*) TEs (Demura et al., 2002; Milioni et al., 2002; Pesquet et al., 2005), and Arabidopsis (Zhao et al., 2005). Despite the abundance of genomic information now available, the function of the large majority of these genes is still unknown.

As compared to Arabidopsis and dicotyledonous plants in general, cell wall research in monocots and, in particular, maize (*Zea mays*) has been much less explored. In the case of primary walls, some isolated examples of the role of cell wall enzymes in relation to specific physiological processes have been reported. For example, the role of cell wall-loosening proteins, including expansins and xyloglucan endotransglycosylase/hydrolases (XTHs), in promoting cell elongation during water deficit has been investigated in maize root tips (Wu et al., 1994, 1996). The inventory of cell wall proteins in the elongation zone of maize roots has recently been enlarged through a systematic proteomic approach (Zhu et al., 2006). Interestingly, although many identified proteins are similar to those previously found in dicot primary walls, many maize wall proteins appear to be specific to monocot walls. It should be possible, at least to some extent, to extrapolate information from cell wall genomics in dicot species, but, considering the profound differences in cell wall structure and composition of monocot and dicot cell walls, it is clear that many biosynthetic and remodeling regulatory mechanisms will not be conserved among these phylogenetically distant taxa. The genomic sequence of rice (*Oryza sativa*) has recently been completed and a comparative approach between cell wall-related gene families in Arabidopsis and rice was undertaken (Yokoyama and Nishitani, 2004). Among the 32 rice cell wall gene families examined, there was a great deal of sequence overlap with cell wall genes in Arabidopsis, but the structural differences in type I (Arabidopsis) versus type II (rice) walls were indeed reflected in certain families. For example, in Arabidopsis pectin is an abundant wall component, whereas in rice it is not. This structural difference was mirrored in the genomic data in that pectin-related gene families were much larger in Arabidopsis than in rice.

At the time of this writing, more than 700,000 ESTs from maize were available. A comparative genomic survey between maize and Arabidopsis revealed that only 60% to 70% of the maize sequences matched with Arabidopsis sequences, indicating a significant proportion of highly diverged or putative maize-specific genes in the maize genome (Brendel et al., 2002). Although global transcriptomic analysis has been undertaken in different physiological contexts, including roots (Poroyko et al., 2005), kernels in response to water stress (Andjelkovic and Thompson, 2006), or organ response to UV light (Casati and Walbot, 2004), an in-depth, systematic characterization of the maize cell wall transcriptome has not been examined (to our knowledge). It should be noted that it is impossible to speak of a maize cell wall per se because the spatial and temporal distribution of wall components is organ, tissue, and cell specific. In general, as stated above, there are several major chemical differences between maize primary cell walls as compared to dicot species (for review, see Carpita and Gibeaut, 1993; Carpita, 1996). Briefly, as is the case in dicots, maize walls contain cellulose microfibrils, but instead of xyloglucan as the main cellulose-tethering molecule, maize is rich in glucuronoarabinoxylans. Maize primary walls are relatively low in pectins, and, based on immunolabeling experiments with antibodies raised against different pectin structures, they appear to be cell type-specific with esterified polygalacturonic acids preferentially localized in vascular tissues, whereas unesterified polygalacturonic acids are mainly found in cortical and parenchyma cells (Knox et al., 1990). The primary walls of maize also contain relatively large amounts of phenolic compounds and little protein in comparison to dicots.

In relation to silage maize digestibility, the lignified secondary cell wall has been extensively studied at the biochemical level (Barrière et al., 2003). However, despite the importance of lignin content and structure in determining forage digestibility, biochemical and molecular regulation of the lignin biosynthetic pathway has been virtually unexplored in maize. That said, expression of a few key genes, including cinnamoyl-CoA reductase (CCR; Pichon et al., 1998), cinnamyl alcohol dehydrogenase (CAD; Halpin et al., 1998), and caffeic acid *O*-methyltransferase (COMT; Capellades et al., 1996), have been spatially and temporally correlated to lignifying tissues. Beyond correlative gene expression data, the function of only two genes, COMT and CAD, has been unambiguously demonstrated (Vignols et al., 1995; Halpin et al., 1998). A well-characterized, naturally occurring mutant, *brown midrib 3* (*bm3*), is mutated in the COMT gene, resulting in less lignin, a decrease in syringyl (S) units, and improved digestibility (Vignols et al., 1995). COMT maize antisense lines were also generated and have a similar, yet less severe, phenotype (Piquemal et al., 2002; He et al., 2003). Similarly, the *bm1* mutant, characterized by altered lignin content and composition, is severely affected in CAD expression (Halpin et al., 1998).

Two specific facts concerning lignification in maize should also be pointed out. First, maize lignin contains relatively high amounts of hydroxycinnamyl (H) units in addition to the guaiacyl (G) and S units typically found in dicotyledonous angiosperms (Lapierre, 1993). This is also the case in most cereals (Lapierre, 1993). Second, maize organs have a wide diversity of lignified cell types. For example, maize internodes are characterized not only by sclerenchyma fibers (located both in the subepidermal cell layers and in close association with vascular bundles) and xylem vessel elements, but also lignified parenchyma cells that represent a very high proportion of the total lignified surface area in older tissues (M. Pichon, unpublished data). Until now, to our knowledge, data have not been made available to integrate knowledge of the specifics of cell wall composition in relation to gene expression in maize.

In this article, we provide a user-friendly, maize cell wall database, MAIZEWALL, containing 735 accessions associated directly or indirectly with primary and secondary wall metabolism. MAIZEWALL is composed of maize homologs resulting from (1) an extensive cell wall-related keyword and BLAST search based on existing knowledge of cell walls in other species; and (2) a BLAST search with ESTs derived from secondary wall-forming in vitro TEs from zinnia (Pesquet et al., 2005). A complete bioinformatic analysis of each gene is provided. A maize cell wall macroarray consisting of gene-specific tags (GSTs), each corresponding to the 3′-untranslated region (UTR) per gene, was constructed. This study provides an organ-specific fingerprint of cell wall-related gene expression in maize. Finally, an in-depth transcriptome analysis of the gene families encoding enzymes of the lignin biosynthetic pathway allowed us to identify putative preferential routes for lignin biosynthesis in different organs and throughout internode development in maize.

RESULTS

MAIZEWALL: A Bioinformatic and Gene Expression Database of Cell Wall Genes in Maize

An overview of the strategy used to construct the cell wall gene catalog found in MAIZEWALL is illustrated in Figure 1. First, a cell wall-related keyword list of nearly 100 words was established based on current knowledge of cell wall synthesis and assembly genes in plants. When available, maize sequences with the appropriate keyword annotation were retrieved from public databases, or, if not, sequences from other plant species were subsequently used as bait to identify the most closely related maize sequences. In this search, we also included genes involved in closely related metabolism (i.e. general phenylpropanoid and shikimic acid pathways) and those controlling vascular patterning that have been identified by the characterization of Arabidopsis mutants (for review, see Scarpella and Meijer, 2004). Second, maize sequences were retrieved based on sequence similarities with zinnia genes expressed during in vitro secondary wall formation (Pesquet et al., 2005). All sequences were then BLASTed against the unannotated maize GénoPlanteInfo (GPI) contig database (Samson et al., 2003) to obtain the corresponding maize contigs. Only contigs with the expected keyword annotation when BLASTed against the public protein databases (SWALL and nonredundant [NR]) were retained. Based on these criteria, 735 contigs were selected as entries for MAIZEWALL (http://www.polebio.scsv.ups-tlse.fr/MAIZEWALL). The 735 contigs belong to 174 putative gene functions, which were further classified into 19 functional categories. The complete cell wall catalog, along with the number of contigs identified for each putative gene function, is found in Supplemental Table S1.

A scheme of the overall structure of the MAIZEWALL database is summarized in Figure 2. Starting

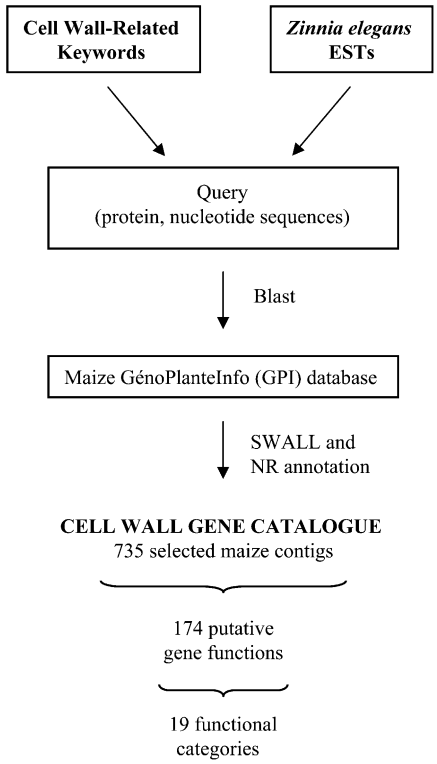


Figure 1. Schematic view of the strategy and content of the cell wall gene catalog found in MAIZEWALL.

with the homepage, the user has direct access to the project description, general information, a versatile sequence search engine, developmental gene expression data, and the cell wall gene catalog. For each gene family found in the gene catalog, detailed bioinformatic analysis has been performed. The user can find contig sequences corresponding mRNA accession numbers and sequences, putative function, and the closest homolog in different plant species, including Arabidopsis and rice, etc. An assortment of bioinformatic software is also provided in a user-friendly interface to perform multiple sequence alignments and identify predicted protein domains and subcellular localization target sequences. Literature references downloaded from PubMed for each family are also available. MAIZEWALL also contains a full set of developmental gene expression data for 651 of the 735 contigs (the difference being that PCR amplification was not successful for all contigs). Gene expression data are accessible either directly from the homepage, as stated above, with genes being ranked from greatest to least expressed per organ and internode stage or from the individual gene family pages. Genes were also clustered to determine those that have overlapping developmental expression profiles. To ensure macroarray signal specificity among gene family members, GSTs based on 3′-UTR sequences were spotted for each contig. Complete details concerning the design and construction of the maize cell wall gene-specific macroarray,

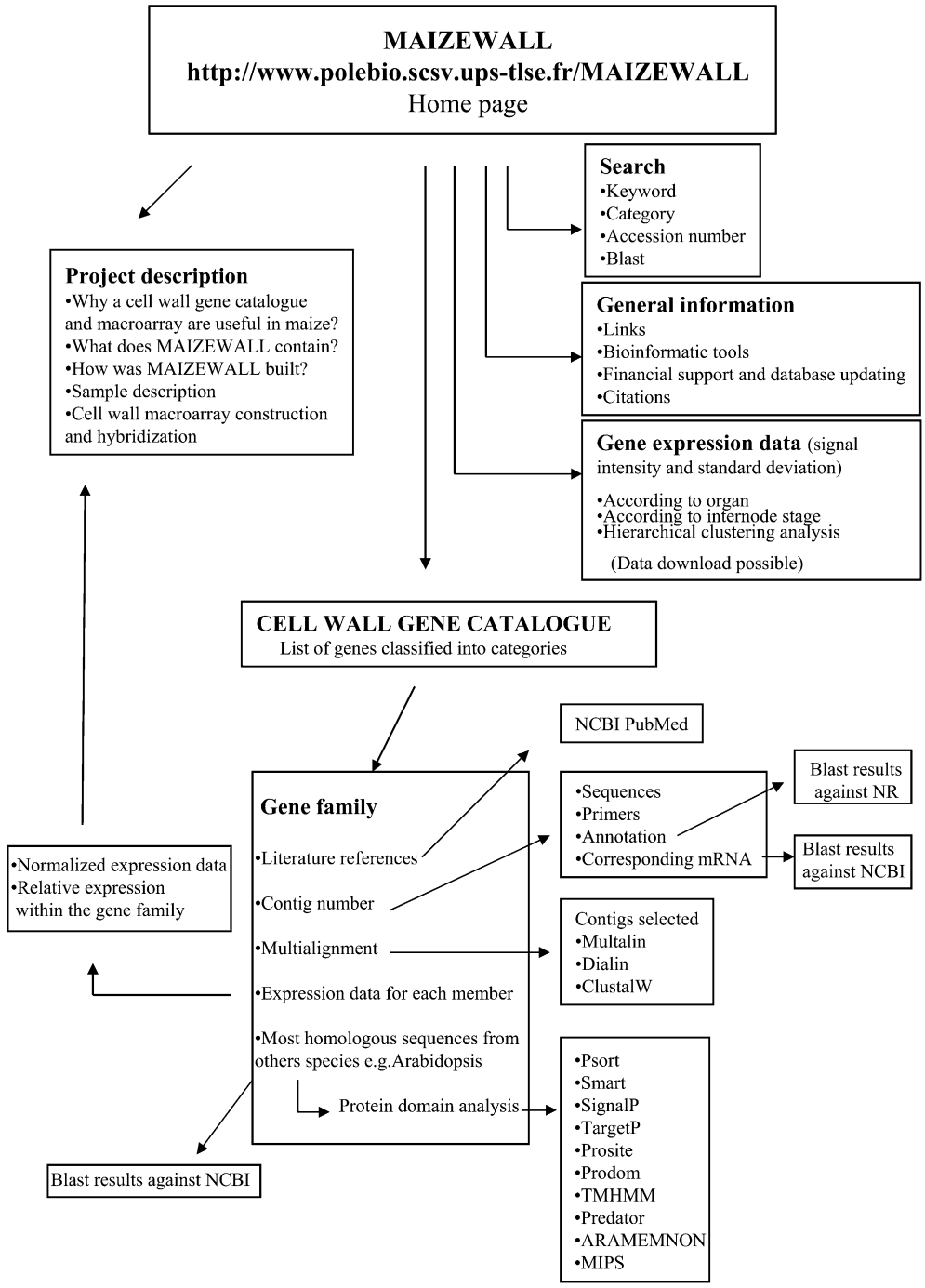


Figure 2. Structure of the MAIZEWALL database. Each box represents a different Web page. Arrows indicate links between pages.

as well as data analysis, can be found in "Materials and Methods" herein or in MAIZEWALL for a more succinct description.

Dynamics of Developmental Cell Wall Gene Expression in Maize

To identify a transcriptional fingerprint of cell wall gene expression in different organs, we compared the global transcript profiles in roots, leaves, and young stems of plants at the four- to five-leaf stage. We first examined the most highly expressed genes, as judged by hybridization signal intensity in each organ (Supplemental Table S2). Data expressed in this manner provide a snapshot of the most highly active metabolic pathways for each organ. Interestingly, when comparing the 30 most highly expressed genes, there is a high degree of overlap among organs (more than one-half), suggesting that the metabolic demands are similar throughout the young maize plant. Not surprisingly, many fall into functional categories related to polysaccharide synthesis. For example, among the 14

cellulose synthase genes spotted on the array, only one contig (QBS7b05.x.g.2.1) is highly expressed in all organs. Along with this cellulose synthase gene, two of the 11 spotted Suc synthase genes are also among the most highly expressed genes, regardless of organ location. These results suggest that the same actors are likely to be important in cellulose synthesis during early maize development. As for hemicelluloses, among the four UDP-Glc-6-dehydrogenase genes, again the same two genes are highly expressed in all organs of young maize plants. These genes encode central enzymes of hemicellulose biosynthesis and appear to be essential for cell wall formation in young organs. Interestingly, some conspicuous differences were observed among the sugar nucleotide-converting enzymes. GDP-Man-4,6-dehydratase (*mur4*) and UDP-D-Xyl-4-epimerase (*mur1*) were among the most highly expressed genes uniquely in the aerial portions of the plant (leaves and young stems).

As a further step toward understanding cell wall gene function, we then searched for genes that exhibited differential expression profiles among organs at the four- to five-leaf stage. A gene was considered differentially expressed when its signal intensity was twice that of one or both of the other two organs examined. Of the 651 GSTs spotted on the array, 180 were not expressed in any organ at this stage of development under our hybridization conditions. Among the remaining 471, 43 were differentially expressed (Table I). Seven genes were preferentially expressed uniquely in young stems and three genes only in roots. Interestingly, there were no cell wall genes exhibiting exclusively leaf-preferential expression. Among the seven genes exhibiting young stem-preferential expression profiles, two of them encoded XTH and three cell wall proteins: a Gly-rich protein (GRP) and two Pro-rich proteins (PRPs). Fourteen were expressed preferentially in both young stems and roots. Among them were four genes encoding enzymes of the phenylpropanoid pathway: two Phe ammonia lyases (PALs), a 4-coumarate: coenzyme A ligase (4CL), and a COMT (Table I). Eight genes were preferentially expressed in the aerial portion of the plant—young stems and leaves. Among them are a *mur1* equivalent and two genes of the phenylpropanoid pathway—a ferulate 5-hydroxylase (F5H) and a CAD.

We then examined the cell wall transcriptome dynamics in internodes at different stages of plant development. Internodes were chosen for this study because they were considered as most suitable to examine both primary and secondary wall gene expression. Global gene expression profiles were obtained at three different stages: piled-up internodes at the four- to five-leaf stage (same as young stems above), and internodes 6 (IN6) and 1 (IN1), which correspond, respectively, to the positions just below the ear and at the base of the plant at silking. Unlike in young plants at the four- to five-leaf stage in which roughly one-third of the spotted genes are not expressed, all of the 651 genes spotted on the array were expressed in at least one internode in this developmental comparison. The 30 most highly expressed genes for each developmental stage are listed in Supplemental Table S3. Certain genes, such as two GRPs, a Suc synthase, and a cellulose synthase, which were already among the 30 most highly expressed in young stems (Supplemental Table S2), remain among the most abundantly expressed genes throughout internode development, whereas others, such as two UDP-Glc-6-dehydrogenases and a UDP-D-Xyl-4-epimerase, a *mur4* equivalent, are only predominant during early internode development, most likely reflecting differences in hemicellulose composition (L. Saulnier, M. Lehaye, M. Pichon, and D. Goffner, unpublished data). In IN6, there is a striking switch of gene expression toward phenylpropanoid metabolism (three PALs, two caffeoyl-CoA *O*-methyltransferases [CCoAOMT], one COMT, and one F5H) and hydroxylation and *O*-methylation enzymes (*S*-adenosyl-Met synthetase 3 and cytochrome P450s), suggesting a high metabolic demand for lignin precursors at this stage of internode development. In IN1, we also noted that a *Medicago truncatula* nodulin 21 (MtN21) homolog was also highly expressed. An analogy may be made with the identification of a MtN21 poplar homolog as one of the most abundant ESTs in a fiber cell death library (Moreau et al., 2005).

We then searched for genes that were differentially expressed at a given moment during internode development. Among the 651 spotted genes, 133 were differentially expressed in at least one stage (Table II). As compared to IN6, very few were preferentially expressed uniquely in young stems or IN1. Of the seven genes expressed in young stems, we detected a pectinesterase and a UDP-D-Gal-4-epimerase, suggesting the importance of pectin modification in the early stages of development. In IN6, many genes involved in phenylpropanoid metabolism are preferentially expressed: two PALs (the two that were among the 30 most highly expressed genes), one 4CL, two CCoAOMTs, one COMT, one CCR, and one CAD). There are also two genes of unknown function (contig nos. 3829406.2.1 and 3071483.2.1) with an extremely high degree of specificity in IN6 as indicated by the relative signal values for the three developmental stages in Table II. When comparing IN6 with young stems, it is interesting to note that different classes of transcription factors (three Myb factors and three monopteros genes) and several members of functionally ill-defined families, such as five callose synthases and three chitinase-like genes, are preferentially expressed. In IN1, lignification has presumably slowed down considerably because there are no phenylpropanoid genes that exhibit preferential expression exclusively at this stage.

Deciphering Developmental Monolignol Biosynthesis throughout the Maize Plant Using Transcriptomics

Despite the economic importance of lignin quantity and quality in dictating certain agronomic traits (Barrière et al., 2003; Méchin et al., 2005), very little is

Table I. *Genes exhibiting preferential expression in organs at the four- to five-leaf stage*

The organ with the highest expression was assigned an arbitrarily value of 1. The others were calculated as a proportion of this value. R, Roots; YS, young stems; L, leaves. As an example, R/YS + L means that gene expression in R is at least twice that of YS and L.

Putative Function	Contig No.	Maize mRNA	Most Homologous Arabidopsis Sequence	Relative Signal Intensity		
				R	YS	L
Preferential expression R/YS + L						
Glucosidase	QCT2a11.yg.2.1	CF060569	At5g42260	1	0.44	0.21
Wall-associated kinase	2440568.2.1	AY108314	At2g47060	1	0.47	0.39
Cys proteinase	2922023.2.1	BT016603	At5g50260	1	0.31	0.32
Preferential expression R/L						
Expansin	AF332180.2.1	AF332180	At1g65680	1	0.55	0.34
Wall-associated kinase	QAF30a10.yg.2.1	CX129506	At1g56145	1	0.73	0.40
Pectinesterase	2448720.2.1	AI629916	At4g12420	1	0.75	0.40
Glucosidase	3062418.2.1	BG320059	At3g18080	1	0.76	0.40
Preferential expression YS/R + L						
XTH	2405117.2.2	BQ744978	At5g57550	0.10	1	0.11
XTH	2591297.2.1	CK348178	At5g13870	0.49	1	0.37
Glucosidase	2306429.2.4	DN205450	At3g18070	0.36	1	0.24
GRP	1716296.2.7	BI361221	At2g21660	0.38	1	0.47
O-Methyltransferase	2440958.2.1	DT938416	At4g35160	0.22	1	0.23
PRP	131537.2.203	AY105945	At4g15160	0.07	1	0.07
Pro-rich AGP-like protein	MAD56_a5f4.3.2	AY105081	At5g22810	0.39	1	0.47
Preferential expression YS/R						
Lipid transfer protein/TE differentiation protein 4 (TED4)	1738978.2.1	BM382382	At3g18280	0.28	1	0.53
O-Methyltransferase	7987759.3.1	DT652416	At4g35160	0.34	1	0.53
UDP-D-Gal-4-epimerase (mur4)	3070703.2.1	BT024099	At4g20460	0.23	1	0.67
Preferential expression YS/L						
Exoglucanase	QCK13d03.yg.3.1	AY103742	At5g20950	0.54	1	0.38
60S ribosomal protein	1804904.2.3	CF017464	At4g27090	0.57	1	0.38
Putative fructokinase II	QBH4g04.xg.3.1	AY197773	At4g10260	0.58	1	0.37
O-Methyltransferase	131572.2.3	AY103669	At4g35150	0.64	1	0.36
Preferential expression YS + L/R						
Emb30	2750663.2.1	BG320107	At1g13980	0.03	0.67	1
Pinoresinol reductase	4424526.2.1	U33318	At4g39230	0.29	0.69	1
GDP-Man-4,6-dehydratase (mur1)	2621786.2.1	CK985788	At5g66280	0.08	0.73	1
F5H1		AX204869	At4g36220	0.04	0.74	1
CAD2	3071507.2.1	DV507972	At4g37980	0.29	1	0.65
Pectinesterase	3115208.2.1	AY103842	At3g13400	0.41	1	0.79
Cytochrome P450	3107376.2.2	AY105992	At2g30750	0.35	1	0.82
PRP	3848939.2.1	CO531431	At3g22120	0.40	1	0.84
Preferential expression YS + R/L						
UDP-GlcUA decarboxylase	1738959.2.3	DR831002	At5g59290	0.58	1	0.27
UDP-GlcUA decarboxylase	1738959.2.2	AY104952	At2g28760	0.63	1	0.24
Argonaute	131537.2.723	CD440332	At5g21150	0.65	1	0.29
Calreticulin	131537.2.334	AY103855	At1g56340	0.66	1	0.34
PRP	2619346.2.2	Y17332	-	0.72	1	0.32
COMT	2192909.2.3	DV551100	At5g54160	0.75	1	0.28
S-adenosyl-Met synthetase 3 (SAMT)	2418879.2.3	BG837557	At1g02500	0.89	1	0.36
PAL/TAL	2161072.2.1	AY103647	At3g53260	1	0.60	0.13
S-adenosyl-Met synthetase 3 (SAMT)	2419471.2.6	BT018468	At4g01850	1	0.66	0.23
DnaJ	2192958.2.2	BT016805	At3g44110	1	0.71	0.33
4CL	3071761.2.1	CF629786	At3g21240	1	0.73	0.35
Ser-Thr kinase (pinoid)	2591032.2.1	DR972540	At5g47750	1	0.78	0.39
Cellulose synthase	2441542.2.1	AF200533	At5g05170	1	0.80	0.37
PAL/TAL	2161072.2.3	BG319893	At3g53260	1	0.99	0.33

known about the monolignol biosynthetic pathway in maize. By mining the maize databases, we have determined that, as is the case in dicots, most of the monolignol biosynthetic enzymes are encoded by multigene families. The first step in assigning a function to each gene family member is to precisely map out expression patterns for each in developmental fashion. In this way, we provide insight into putative preferred routes of monolignol biosynthesis throughout the maize plant. For each of the monolignol

Table II. *Genes exhibiting preferential expression at different stages of internode development*

The internode with the highest expression was arbitrarily assigned a value of 1. The others were calculated as a proportion of this value. YS, Young stems. As an example, YS/IN6 + IN1 means that gene expression in YS is at least twice that of IN6 and IN1.

Putative Function	Contig No.	Maize mRNA	Most Homologous Arabidopsis Sequence	Relative Signal Intensity		
				YS	IN6	IN1
Preferential expression YS/IN6 + IN1						
UDP-D-Gal-4-epimerase (mur4)	3070703.2.1	BT024099	At4g20460	1	0.28	0.25
CCoAOMT	2591258.2.1	DT643982	At4g34050	1	0.36	0.50
Chalcone isomerase	3390529.2.1	AW157942	At3g55120	1	0.29	0.44
Endonuclease	2823202.2.1	AI783233	At1g68290	1	0.39	0.27
Pectinesterase	3115208.2.1	AY103842	At3g13400	1	0.42	0.29
Unknown gene (close to DV017580)	5704580.2.1	DN231044	At1g71820	1	0.29	0.48
Glucosyl transferase	3748389.2.1	AW355881	At2g22590	1	0.38	0.50
Preferential expression YS/IN6						
Arabinogalactan protein (AGP)	8635788.2.1	BG840766	At5g03170	1	0.38	0.52
Chorismate mutase	2521459.2.4	DN204889	At1g69370	1	0.34	0.61
Integral membrane protein (cov-1)	3562100.2.1	DY536894	At2g20120	1	0.33	0.55
Endo-1,3-1,4-β-D-glucanase	3713002.2.1	AY109289	At4g16260	1	0.31	0.58
Glucosidase	2763158.2.1	AY106297	At5g44640	1	0.37	0.58
Laccase	8616263.2.1	BG842157	At5g60020	1	0.29	0.51
Laccase	2440419.2.1	AI491689	At5g21100	1	0.40	0.63
Pectate lyase	3198766.2.1	EB402435	At1g67750	1	0.28	0.55
Ser-Thr kinase (pinoid)	2591032.2.1	DR972540	At5g47750	1	0.33	0.57
PRP	1738863.2.3	DT943178	At2g10940	1	0.35	0.57
SHP1 MADS-BOX	L46398.2.1	L46398	At2g45650	1	0.39	0.52
Syntaxin-related protein KNOLLE	2750436.2.1	DN225275	At1g08560	1	0.32	0.54
ABC transporter	3024030.2.1	DT653269	At2g28070	1	0.39	0.56
ADP/ATP carrier protein	131537.2.472	AY108334	At4g01100	1	0.37	0.59
Expansin	3696569.2.1	AF332173	At1g69530	1	0.37	0.58
Argonaute	131537.2.170	CD441197	At5g21030	1	0.35	0.53
Cucumisin	7297169.2.1	BF727798	At3g14067	1	0.28	0.54
Cys proteinase	3173057.2.1	AY106278	At5g43060	1	0.38	0.61
MtN21 nodulin-like protein	3203235.2.1	AW065996	At5g07050	1	0.34	0.53
Pectinesterase	2448720.2.1	AI629916	At4g12420	1	0.40	0.63
Peroxidase	2619325.2.1	AJ401275	At5g05340	1	0.28	0.53
S-adenosyl-L-Met:salicylic acid carboxyl methyltransferase	QBL17f07.xg.2.1	DR830682	At4g36470	1	0.37	0.52
Preferential expression YS/IN1						
Plasma membrane protein (kobito1-2)	QAF31c07.yg.2.3	DN220113	At3g08550	1	0.63	0.39
Ubiquitin-conjugating enzyme E2	1716236.2.1	EB400137	At1g64230	1	0.62	0.37
Suc synthase	QAS4c05.yg.2.1	CB179646	–	1	0.60	0.37
PGPD14 protein	3198841.2.2	AW331066	At5g22920	1	0.60	0.40
CAD2	4424417.2.1	AY110917	At4g37980	1	0.60	0.37
Lipid transfer protein/TE differentiation protein 4 (TED4)	2418885.2.3	AY104780	At5g38170	1	0.57	0.41
Polygalacturonase	3696657.2.1	AY110071	At2g33160	1	0.57	0.35
Pectinesterase	2404725.2.9	DT940712	At3g13400	1	0.55	0.40
Small nuclear ribonucleoprotein	131584.2.4	DR818246	At2g47640	1	0.52	0.38
Preferential expression YS + IN6/IN1						
CCoAOMT	2455940.2.1	AJ242980	At4g34050	0.74	1	0.36
Glucosidase	2306429.2.4	DN205450	At3g18070	1	0.90	0.37
Benzoquinone reductase-like	2441114.2.1	BT017374	At5g54500	1	0.84	0.40
F5H1		AX204869	At4g36220	1	0.84	0.25
Dolichyl-di-phosphooligosaccharide-protein glycotransferase	131537.2.569	DR970321	At5g66680	1	0.81	0.40
Calmodulin	2441373.2.3	DV520027	At3g43810	1	0.79	0.40
O-Methyltransferase	131572.2.3	AY103669	At4g35150	1	0.75	0.21
GDP-Man 4,6-dehydratase (mur1)	2621786.2.1	CK985788	At5g66280	1	0.70	0.24
Emb30	2750663.2.1	BG320107	At1g13980	1	0.63	0.30
PRP	131537.2.203	AY105945	At3g22120	1	0.58	0.10

(*Table continues on following page.*)

Table II. (*Continued from previous page.*)

Putative Function	Contig No.	Maize mRNA	Most Homologous Arabidopsis Sequence	Relative Signal Intensity		
				YS	IN6	IN1
Preferential expression IN6/YS + IN1						
Alcohol dehydrogenase	QAY3d01.yg.3.1	EB399902	At1g49670	0.36	1	0.49
4CL	3071761.2.1	CF629786	At3g21240	0.33	1	0.27
CCR	3012873.2.2	Y13734	At1g15950	0.44	1	0.30
CCoAOMT	2430769.2.1	AW231479	At4g34050	0.40	1	0.26
CCoAOMT	2455940.2.2	AJ242981	At4g34050	0.41	1	0.42
Callose synthase	131612.2.1	AI692047	At5g13000	0.38	1	0.35
Cellulose synthase-like (Csl)	2568371.2.1	AI673968	At5g16910	0.40	1	0.44
Chalcone isomerase	2647051.2.1	DV550165	At3g55120	0.45	1	0.30
Expansin	2478337.2.1	CO532987	At2g45110	0.32	1	0.36
Exoglucanase	2762970.2.1	DT642729	At5g20950	0.38	1	0.50
Glucosidase	2750951.2.1	BE640554	At3g18080	0.33	1	0.49
Glucosidase	QCT2a11.yg.2.1	CF060569	At5g42260	0.45	1	0.18
COMT	2192909.2.3	DV551100	At5g54160	0.31	1	0.28
Pinoresinol reductase	4424526.2.1	U33318	At4g39230	0.50	1	0.37
PAL/TAL	2161072.2.3	BG319893	At3g53260	0.19	1	0.16
PAL/TAL	2161072.2.1	AY103647	At3g53260	0.11	1	0.20
S-adenosyl-Met synthetase 3 (SAMT)	2418879.2.3	BG837557	At1g02500	0.34	1	0.33
CAD2	3203838.2.1	CD995201	At4g39330	0.38	1	0.46
Xylosidase	5532690.2.1	DV622656	At5g10560	0.38	1	0.45
XTH	2405117.2.2	BQ744978	At5g57550	0.49	1	0.11
Benzoquinone reductase-like	2441114.2.2	AY104807	At5g54500	0.36	1	0.26
Cys proteinase	2922023.2.1	BT016603	At5g50260	0.42	1	0.37
Dolichyl-di-phosphooligosaccharide-protein glycotransferase	131537.2.288	AY103792	At5g66680	0.42	1	0.41
Oligopeptide transporter	3204486.2.1	DR957852	At3g54140	0.32	1	0.44
Proteinase inhibitor/γ-thionin	2494085.2.2	DV495756	At2g02100	0.34	1	0.48
Unknown gene (close to DV017148)	3829406.2.1	CF624874	At5g05960	0.07	1	0.11
Unknown gene (close to DV017148)	3071483.2.1	AI948098	At5g05960	0.08	1	0.10
Expansin	QBJ24c04.pg.2.1	AW566451	At1g65680	0.34	1	0.46
MtN21 nodulin-like protein	QBTB.068P15F020924.3.1	DT648847	At3g18200	0.40	1	0.48
Transcription regulatory protein	QAF18e02.yg.3.1	CD435681	At1g02080	0.35	1	0.50
Histone promoter-binding protein HBP-1A(C14) transcription factor	QBTB.064G21F020918.3.1	DR794027	At4g36730	0.37	1	0.50
Preferential expression IN6/YS						
MYB transcription factor	QBS9a01.xg.2.1	DR809108	At4g01680	0.38	1	0.72
MYB-like transcription factor (APL)	5110719.2.1	DV550965	At2g01060	0.37	1	0.62
Callose synthase	QCH10g11.yg.2.1	AC185276	At4g04970	0.35	1	0.51
Callose synthase	QCG38e04.yg.2.1	CX129552	At5g13000	0.29	1	0.53
Callose synthase	3148257.2.1	AW090958	At4g04970	0.38	1	0.54
Callose synthase	QCG13d12.yg.2.1	AY107727	At5g13000	0.40	1	0.61
Callose synthase	QCN28g01.yg.2.1	DV172790	At2g31960	0.33	1	0.65
Cellulose synthase-like (Csl)	QAH2a02.xg.2.1	AX756398	At3g03050	0.40	1	0.53
Chalcone synthase	3802552.2.1	AW400268	At5g13930	0.37	1	0.63
Chitinase-like	4679577.2.1	DR819933	At3g54420	0.36	1	0.53
Emb30	QCH30c02.yg.2.1	AI691537	At1g13980	0.36	1	0.57
XTH	3838024.2.1	BF728605	At5g57560	0.41	1	0.57
Expansin	2649891.2.1	AI740146	At2g45110	0.37	1	0.69
Galactosyltransferase	QCN21b10.yg.2.1	CK369130	At4g21060	0.40	1	0.53
Galactosyltransferase	QCH13e05.yg.2.1	DY537801	At1g26810	0.40	1	0.60
Glucosidase	QAY3d02.yg.2.1	DR814197	At1g61820	0.36	1	0.56
GRP	1716296.2.7	BI361221	At2g21660	0.39	1	0.72
Cellulose synthase-like	QCL24b11.yg.2.1	BM380526	At2g21770	0.37	1	0.52
Transcription factor (monopteros)	2750753.2.1	AY106228	At5g62000	0.36	1	0.51
Transcription factor (monopteros)	QCS19f07.yg.2.1	CF059990	At1g19220	0.37	1	0.53
Transcription factor (monopteros)	QCG16f05.yg.2.1	CF035906	At2g33860	0.36	1	0.58
MYB transcription factor	QBL16c05.xg.2.1	DY686346	At1g09540	0.34	1	0.52
Pectin methyl esterase	3712967.2.1	BF728155	At2g47550	0.38	1	0.64
Ser-Thr kinase (pinoid)	4534747.2.1	BE012117	At3g12690	0.38	1	0.53

(*Table continues on following page.*)

Table II. (*Continued from previous page.*)

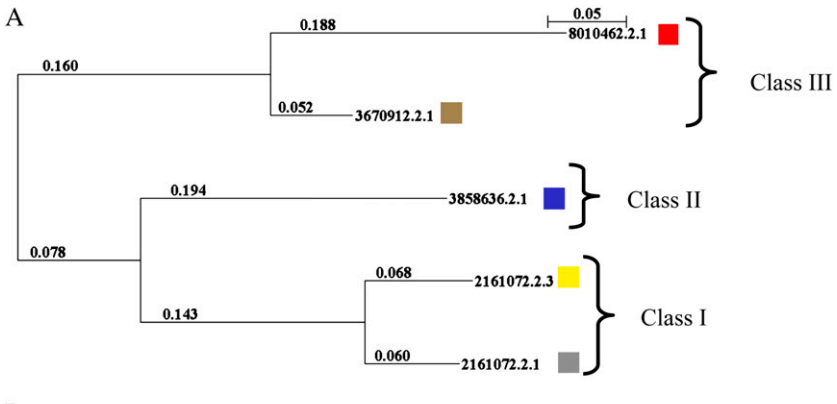
Putative Function	Contig No.	Maize mRNA	Most Homologous Arabidopsis Sequence	Relative Signal Intensity		
				YS	IN6	IN1
Putative glycosyltransferase (quasimodo)	3642920.2.1	AW261329	At5g15470	0.40	1	0.59
Putative glycosyltransferase (quasimodo)	3147935.2.1	CF039403	At5g47780	0.36	1	0.59
Putative glycosyltransferase (quasimodo)	3064721.2.2	DR958277	At5g47780	0.38	1	0.63
Aldehyde dehydrogenase/reduced epidermal fluorescence (REF1)/Restore fertility (RF2)	2621801.2.1	DR822602	At1g23800	0.32	1	0.56
SHP1 MADS-BOX	3829479.2.1	L81162	At4g18960	0.39	1	0.55
Sterol methyltransferase	3748535.2.1	AW356027	At5g13710	0.39	1	0.59
Wall-associated kinase	QCG22g05.yg.2.1	CS226436	At2g37050	0.39	1	0.63
Preferential expression IN6/IN1						
CAD2	3071507.2.1	DV507972	At4g37980	0.64	1	0.37
PAL/TAL	3858636.2.1	CF631905	At3g53260	0.52	1	0.30
PRP	2419137.2.4	AI857154	At1g62510	0.57	1	0.33
Peroxidase	2440918.2.1	AY106450	At1g05260	0.60	1	0.36
Chalcone synthase	QBTB.065K11F020919.3.1	DT640793	At5g13930	0.58	1	0.38
Preferential expression IN6 + IN1/YS						
ATHB-8 HD-zip protein	2521569.2.1	DR784986	At1g52150	0.21	1	0.58
Glutathione *S*-transferase (Bronze-2)	3713091.2.1	AF244704	At1g10370	0.30	1	0.63
Chitinase-like	131537.2.387	BG837630	At3g12500	0.26	1	0.64
Chitinase-like	131537.2.593	BG837392	At3g12500	0.27	1	0.73
Abscisic acid-induced protein	2478113.2.1	CF024514	At5g50720	0.34	1	0.85
GRP	131537.2.78	CD441567	At2g21660	0.29	0.64	1
Preferential expression IN1/YS + IN6						
GRP	131537.2.623	AF034945	At2g21660	0.33	0.50	1
Extensin	QCT19a08.yg.2.1	DR806234	At4g13340	0.23	0.23	1
Cytochrome P450	1716424.2.3	AY072298	At3g26280	0.39	0.35	1
Preferential expression IN1/IN6						
MtN21 nodulin-like protein	3995289.2.1	AW562846	At1g75500	0.53	0.39	1
Preferential expression IN1 + YS/IN6						
Arabinogalactan protein (AGP)	AB021175.2.1	AB021175	At3g19430	1	0.23	0.54
Arabinogalactan protein (AGP)	AB021176.2.1	AB021176	At3g19430	1	0.25	0.57
Glutathione *S*-transferase (Bronze-2)	3829517.2.1	AF244682	At2g02390	1	0.24	0.53
Cell wall invertase	2943856.2.2	U17695	At3g52600	1	0.20	0.52
Endo-1,3-1,4-β-D-glucanase	2493751.2.1	DR797601	At4g26830	1	0.23	0.53
UDP-Glc-6-dehydrogenase	2750995.2.1	CF024455	At3g29360	1	0.25	0.51
Dihydroflavonol reductase	3184927.2.1	AY109666	At4g35420	0.72	0.31	1

biosynthetic pathway genes, a complete enzyme-by-enzyme survey of developmental expression patterns in different organs and throughout internode development is described below.

PAL/Tyr Ammonia Lyase

PAL catalyzes the first step in the phenylpropanoid pathway by removing ammonia from L-Phe to produce *p*-coumaric acid. In maize, PAL also has Tyr ammonia lyase (TAL) activity in that the enzyme utilizes Tyr in addition to Phe as substrate (Rösler et al., 1997). In all plants analyzed thus far, PAL is encoded by multigene families. In the complete genome of Arabidopsis, four genes encoding PAL proteins have been identified (Raes et al., 2003). In maize, one PAL/TAL cDNA has been previously reported, but no data on gene number or expression patterns are available (Rösler et al., 1997).

Using the sequence previously described by Rösler et al. (1997), a total of five contigs annotated PAL/TAL were identified with nucleic acid sequence identities ranging from 57% to 84% among them (Supplemental Fig. S4A). Phylogenetic analysis allowed us to define three classes: classes I, II, and III (Fig. 3A). Class I contains two sequences: the one originally described by Rösler et al. (1997) (contig no. 2161072.2.1) and another with 84% identity at the nucleic acid level (contig no. 2161072.2.3). Class II contains only one member, and class III contains two members that exhibit 77% identity between them. Based uniquely on sequence identity, we were unable to establish an unambiguous relationship with other previously described PAL genes from other species. For example, the class I maize PAL (contig no. 2161072.2.3) exhibited 64% and 66% sequence identity at the protein level with Arabidopsis PAL1 and PAL2, respectively. On the other hand, the same maize contig exhibited 89% and 71% identity at the protein level with rice PAL1 and PAL2, respectively, suggesting that this maize gene is most likely the rice PAL1 putative ortholog (Zhu et al., 1995).



B

	Roots	Leaves	Young Stem	Internode 6	Internode 1
8010462.2.1	8002	-	8555	10421	-
3670912.2.1	-	6177	7180	9192	-
3858636.2.1	49232	30542	53856	102659	31100
2161072.2.3	39091	12977	38758	207907	32836
2161072.2.1	34483	-	20523	187353	38154
Total	130808	49696	128872	517532	102090

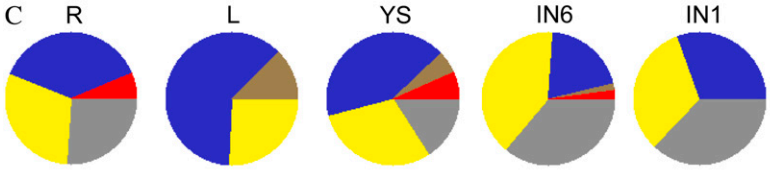


Figure 3. Characterization of the PAL gene family in maize. A, Phylogenetic analysis. Classes were defined according to evolutionary distances defined by Phylip 3.5 software. B, Gene expression in organs at the four- to five-leaf stage and IN1 and IN6 at silking. Values indicate the normalized signal hybridization intensity for each gene and (−) signifies a below-background signal intensity of ≤6,000. C, Relative contribution of each gene to total PAL gene expression. R, Roots; L, leaves, YS, young stems, which results from piled-up internodes at the four- to five-leaf stage. IN6, Internode just below the node bearing the ear at silking; IN1, basal internode at silking. Note that equivalent datasets as found in B and C are available for all gene entries in MAIZEWALL.

The gene expression of each family was first analyzed globally by summing up the hybridization signal intensities corresponding to all of the identified family members for each organ and stage of internode development. This provides a means to assess total transcriptional activity for the entire gene family per organ and developmental stage. The relative contribution of each family member was also systematically evaluated by determining the percentage of contribution toward total measured transcriptional activity. As for the different organs of young plants, the highest global PAL gene expression was observed in stems and roots, with relatively low expression level in leaves (Fig. 3B). A class I PAL (contig no. 2161072.2.3) was expressed in relatively equal proportions in the three organs, whereas the other class I member (contig no. 2161072.2.1) was moderately expressed in stems and roots, but totally absent in leaves. Interestingly, the class II PAL gene (contig no. 3858636.2.1) was proportionally the most highly expressed of all the PAL genes in all three young organs. The two class III PAL members have little to no expression in young plants (Fig. 3C). When considering the different stages of internode development, global PAL gene family expression was highest in IN6 (Fig. 3B). Whereas the class II member (contig no. 3858636.2.1) was predominant in young stems, the two class I PALs (contig nos. 2161072.2.3 and 2161072.2.1) were, by far, the most highly expressed in IN6. As is the case for young organs, class III members show little to no expression, even in IN6 and IN1.

Cinnamate 4-Hydroxylase

In conjunction with two other key enzymes of the core phenylpropanoid pathway, PAL and 4CL, cinnamate 4-hydroxylase (C4H) directs carbon flux into phenylpropanoid metabolism. C4H belongs to the CYP73A group of the cytochrome P450 family and catalyzes the first oxidative reaction in phenylpropanoid metabolism, namely, the conversion of trans-cinnamic to *p*-coumaric acid. This reaction consumes molecular oxygen and a reducing equivalent from NADPH delivered via cytochrome P450 reductase (Meijer et al., 1993). In Arabidopsis, C4H is encoded by one gene, which is expressed in all tissues (Mizutani et al., 1997). In maize, we identified two sequences, C4H1 corresponding to contig number 2521589.2.1 and C4H2 cloned by reverse transcription (RT)-PCR in our laboratory. They exhibit 72% identity between

them at the nucleotide level. At the protein level, both C4H1 and C4H2 from maize exhibited 75% identity/86% similarity and 81% identity/91% similarity, respectively, with Arabidopsis C4H. Both genes are expressed in all organs and at all stages of internode development (Table III). C4H1 (contig no. 251589.2.1) is by far the predominant gene in IN6. C4H2, on the other hand, has a tendency to be more highly expressed in all organs of young plants.

4CL

4CL, which catalyzes the formation of CoA esters of *p*-coumaric acid, caffeic acid, ferulic acid, 5-hydroxyferulic acid, and sinapic acid, plays a pivotal role in channeling phenylpropanoid precursors into different downstream pathways, each leading to a variety of functionally distinct end products (Harding et al., 2002). This probably explains why 4CL is encoded by relatively large multigene families. 4CL isoforms in Arabidopsis have been extensively characterized at the biochemical level (Hamberger and Hahlbrock, 2004; Costa et al., 2005) and their gene expression profiles analyzed (Raes et al., 2003).

To date, only two sequences have been reported in maize (Puigdomenech et al., 2001; S. Sivasankar, D. Sapienza, and T. Helentjaris, unpublished data). Using these maize sequences as bait, we retrieved a total of seven contigs (Fig. 4A). The percentage of nucleic acid identity among the sequences ranges from 43% to 82% (Supplemental Fig. S4B). Among them, a class I member (contig no. 3071761.2.1) corresponding to the maize sequence isolated by S. Sivasankar, D. Sapienza, and T. Helentjaris (unpublished data) is a putative ortholog to 4CL2 from Arabidopsis (Hamberger and Hahlbrock, 2004) and poplar (Hu et al., 1998).

From a quantitative standpoint, global 4CL expression is quite similar in all organs of young plants (Fig. 4B). A closer examination of each family member indicated that the Arabidopsis 4CL2 homolog (contig no. 3071761.2.1) is moderately expressed in young roots and stems, but absent in leaves (Fig. 4C). The class II 4CL gene is constitutively expressed at high levels in all young organs, but is the dominant form in leaves. This gene is most homologous to 4CL1 from Arabidopsis. Interestingly, a class IV 4CL (contig no. 171632.2.2) appeared to be specific to the aerial portions of a young plant, whereas a class III 4CL (3106166.2.1) that is most homologous to 4CL-like7 in Arabidopsis is the major form expressed in roots. 4CL gene expression was then monitored during internode development. Global 4CL expression is extremely high in IN6, with all of the 4CL genes expressed at this stage of development. The class I 4CL homolog to 4CL2 in Arabidopsis (contig no. 3071761.2.1) is the predominant 4CL gene in IN6.

Table III. *Hybridization signal intensities of lignification genes encoded by one or two genes*

Numbers in this table represent normalized signal intensity; (−) signifies a below-background signal intensity of ≤6,000; pcr signifies that the cDNA spotted on the macroarray resulted from RT-PCR.

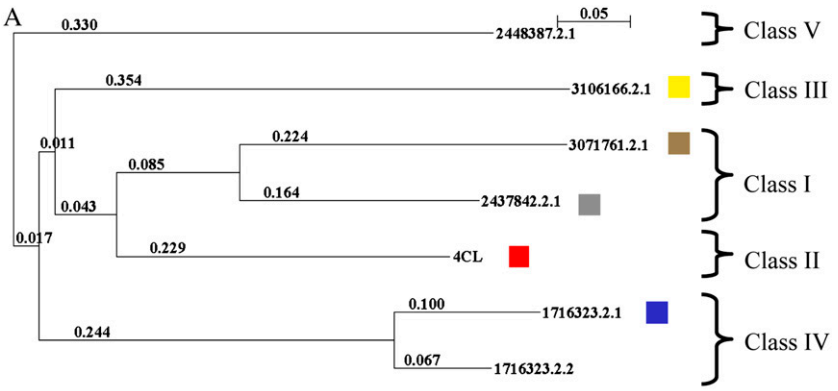
Genes	Contig No.	Roots	Young Stem	Leaves	IN6	IN1
C4H1	2521589.2.1	41,797	28,668	23,250	66,372	28,254
C4H2	pcr	57,838	34,655	38,742	19,988	30,822
C3H	2643622.2.1	6,280	7,892	8,132	10,568	7,621
HCT1	2478084.2.1	–	–	–	11,068	–
HCT2	2619423.2.1	10,901	11,005	6,868	14,343	9,178
F5H1	pcr	–	54,198	73,698	45,662	13,754
COMT	2192909.2.3	32,664	43,536	12,054	142,202	39,112

Hydroxycinnamoyl-CoA Transferase

Hydroxycinnamoyl-CoA transferase (HCT) is the most recently identified actor in monolignol biosynthesis and belongs to a large family of acyltransferases (Hoffmann et al., 2003). It catalyzes the conversion of *p*-coumaroyl-CoA and caffeoyl-CoA to their corresponding shikimate or quinate esters just up and downstream of *p*-coumarate 3-hydroxylase (C3H). In Arabidopsis, only one gene has been detected in the genome and it is expressed in all tissues investigated, with the strongest expression in inflorescence stems (Raes et al., 2003). In maize, we identified two HCT contigs with 64% identity between them at the nucleotide level. HCT1 (contig no. 2478084.2.1) exhibited 52% identity/63% similarity at the protein level with HCT originally isolated from tobacco (*Nicotiana tabacum*; Hoffmann et al., 2003). HCT2 (contig no. 2619423.2.1) exhibited 58% identity/68% similarity with the tobacco protein. HCT2 (contig no. 2619423.2.1) is expressed at relatively low levels in all young organs and throughout internode development, whereas HCT1 (contig no. 2478084.2.1) is only expressed in IN6 (Table III).

C3H

It was originally postulated that this enzyme catalyzed the C3 hydroxylation step from *p*-coumaric to caffeic acid. More recently, it has been shown that C3H preferentially converts the shikimate and quinate esters of *p*-coumaric acid into their corresponding caffeic acid conjugates (Schoch et al., 2001; Franke et al., 2002). C3H belongs to the CYP98 cytochrome P450 family. In Arabidopsis, three C3H genes have been identified in the genome (Raes et al., 2003). One of them, C3H1, clusters with all known C3Hs from other species, whereas C3H2 and C3H3 are more divergent and constitute a separate class and do not appear to hydroxylate shikimate and quinate esters of *p*-coumaric acid (Schoch et al., 2001). In maize, we found only one contig (no. 2643622.2.1) that annotated C3H (Table III). The sequence exhibited 65% identity with the C3H1 Arabidopsis gene, with much lower homology to C3H2 and C3H3 (49% identity with each). In maize, C3H exhibited relatively low levels of expression in all organs studied, with slightly higher levels in IN6.



B

	Roots	Leaves	Young Stem	Internode 6	Internode 1
3106166.2.1	19184	8804	13873	10286	10709
3071761.2.1	10139	-	7409	22522	6154
2437842.2.1	-	-	6711	10324	-
4CL	17773	21020	15450	10707	11347
1716323.2.1	-	7873	10565	8181	-
Total	47096	37697	54008	72727	28210

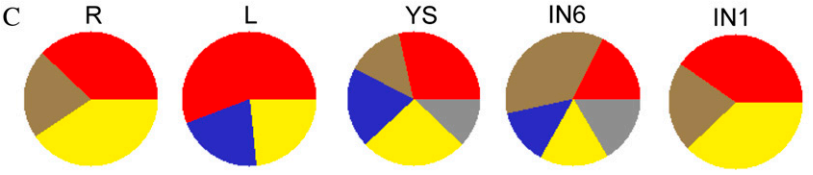


Figure 4. Characterization of the 4CL gene family in maize. A, Phylogenetic analysis. Classes were defined according to evolutionary distances defined by Phylip 3.5 software. Contig numbers 2448387.2.1 and 17163.2.2 annotated as 4CL-like; contig numbers 3106166.2.1 and 1716323.2.1 annotated as putative 4CL. For 4CL2, a contig was not found in the GPI databases. B, Gene expression in organs at the four- to five-leaf stage and internodes at different developmental stages. Values indicate the normalized signal hybridization intensity for each gene and (−) signifies a below-background signal intensity of ≤6,000. Note that contig numbers 1716323.2.2 and 2448387.2.1 were not included in expression studies because we were unable to amplify corresponding GSTs. C, Relative contribution of each gene to total 4CL gene expression. R, Roots; L, leaves; YS, young stems, which results from piled-up internodes at the four- to five-leaf stage. IN6, Internode just below the node bearing the ear at silking; IN1, basal internode at silking. Note that equivalent datasets as found in B and C are available for all gene entries in MAIZEWALL.

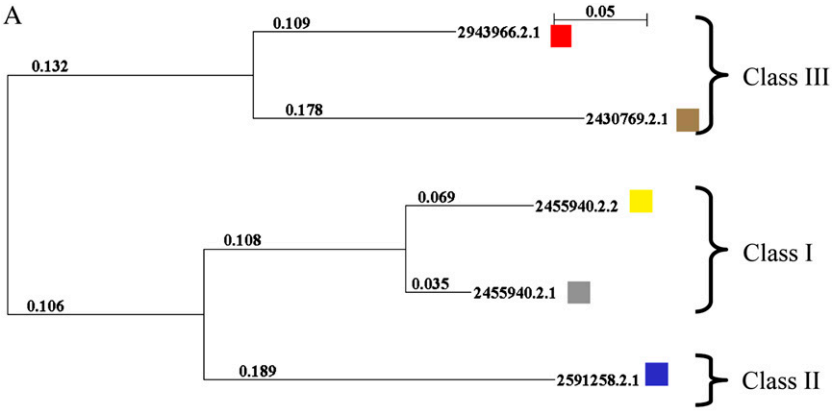
CCoAOMT

CCoAOMT, by catalyzing the methylation of caffeoyl-CoA to feruloyl-CoA and 5-hydroxyferuloyl-CoA to sinapoyl-CoA, plays a pivotal role in determining lignin composition (Zhong et al., 1998; Pinçon et al., 2001). Seven CCoAOMT gene family members with different spatiotemporal gene expression patterns were identified in the Arabidopsis genome (Raes et al., 2003). In maize, two CCoAOMT sequences, CCoAOMT1 and CCoAOMT2, have been previously deposited in public databases (Civardi et al., 1999), but to our knowledge no expression data for these genes have ever been reported. By BLASTing these two sequences in GPI databases, we were able to identify a total of five contigs that fell into three classes (Fig. 5A). Class I contains two members (contig nos. 2455940.2.1 and 2455940.2.2) that correspond to the previously identified CCoAOMT1 and CCoAOMT2, respectively. They exhibited 83% identity at the nucleotide level (Supplemental Fig. S4C). Class II contains one member (contig no. 2591258.2.1) and class III contains two members (contig nos. 2943966.2.1 and 2430769.2.1). In general, global CCoAOMT expression was similar in all organs studied, except in IN1, where expression was significantly lower (Fig. 5B). In all young organs, although all five genes are constitutively expressed, the class II member (2591258.2.1) is the most highly expressed (Fig. 5, B and C). At silking, although all five genes are still expressed in IN6 and IN1, contig number 2455940.2.2 (CCoAOMT2) and contig number 2430769.2.1 account for a large majority of CCoAOMT expression in IN6. It should be noted that CCoAOMT1 (contig no. 2455940.2.1) is expressed at low levels in all organs examined, suggesting that it is not the major actor in lignin biosynthesis in maize. In Arabidopsis, among the seven putative CCoAOMT genes, based on expression patterns, CCoAOMT1 was considered to be the gene most likely involved in constitutive lignification (Raes et al., 2003). Based on sequence homology alone, it is impossible to determine whether CCoAOMT1 or CCoAOMT2 is the functional maize equivalent of this Arabidopsis gene. In any case, based on expression data, CCoAOMT2 appears to be more important for constitutive lignification in maize as compared to CCoAOMT1.

CCR

CCR catalyzes the conversion of hydroxycinnamoyl-CoA esters (*p*-coumaroyl-CoA, feruloyl-CoA,

A



B

	Roots	Leaves	Young Stem	Internode 6	Internode 1
2943966.2.1	28246	17730	21850	13947	14245
2430769.2.1	25082	14124	20497	51550	13430
2455940.2.2	22054	15606	19316	47434	19746
2455940.2.1	8823	9016	16255	21976	7869
2591258.2.1	68367	57352	65846	23740	32846
Total	152571	113828	143763	158647	88137

C

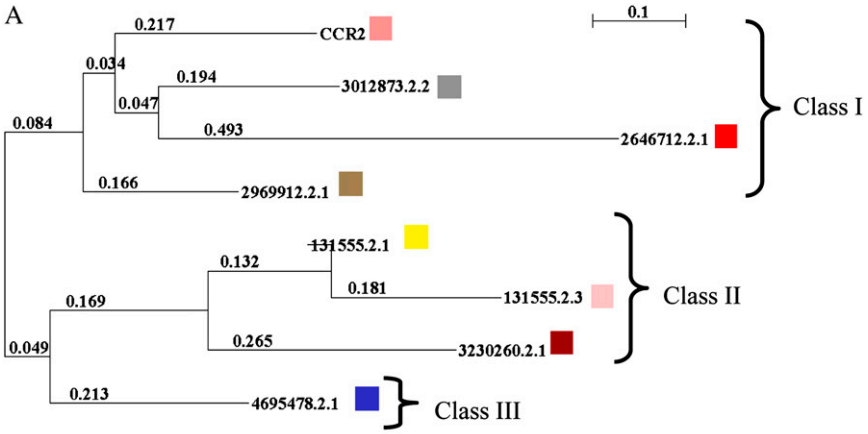
Figure 5. Characterization of the CCoAOMT gene family in maize. A, Phylogenetic analysis. Classes were defined according to evolutionary distances defined by Phylip 3.5 software. Contig numbers 2591258.2.1, 2430769.2.1, and 2943966.2.1 annotated as putative CCoAOMT. B, Gene expression in organs at the four- to five-leaf stage and internodes at different developmental stages. Values indicate the normalized signal hybridization intensity for each gene. C, Relative contribution of each gene to total CCoAOMT gene expression. R, Roots; L, leaves; YS, young stems, which results from piled-up internodes at the four- to five-leaf stage. IN6, Internode just below the node bearing the ear at silking; IN1, basal internode at silking. Note that equivalent datasets as found in B and C are available for all gene entries in MAIZEWALL.

sinapoyl-CoA) into their corresponding cinnamyl aldehydes and is therefore the first committed enzyme of the monolignol pathway. Two full-length cDNAs, ZmCCR1 and ZmCCR2, have previously been cloned and their expression pattern in different organs characterized (Pichon et al., 1998). ZmCCR1 was highly expressed along the stalk, suggesting that the corresponding enzyme was probably involved in constitutive lignification. Using ZmCCR1 to BLAST the GPI database, in addition to ZmCCR1 (contig no. 3012873.2.2), six other contigs annotated as CCR/putative CCR were identified (Fig. 6). According to phylogenetic analysis, the seven CCR/putative CCRs formed three classes (Fig. 6A). CCRs exhibited 39% to 59% identity with ZmCCR1 (contig no. 3012873.2.2) and 38% to 83% among all family members (Supplemental Fig. S4D).

In young plants, global CCR expression is higher in roots and stems than in leaves (Fig. 6B). Many CCR gene family members are not expressed during early plant development, with essentially two major genes accounting for the large majority of overall CCR expression in all young organs: ZmCCR1 (3012873.2.2) and the class III putative CCR (contig no. 4695478.2.1). In keeping with previously reported data, CCR2 is mainly expressed in roots (Pichon et al., 1998). During internode development, global CCR expression was maximal in IN6 (Fig. 6B). Although all CCR and putative CCR genes are expressed at this stage, ZmCCR1 is expressed to a much greater extent than all the others (Fig. 6C).

F5H

F5H, or coniferaldehyde 5-hydroxylase, is a cytochrome P450-dependent monooxygenase (CYP84) and a key enzyme for the production of S-unit lignin in dicotyledonous angiosperms (Humphreys et al., 1999). It catalyzes the 5-hydroxylation of coniferaldehyde and/or coniferyl alcohol. In Arabidopsis, two genes encoding F5H have been identified (Raes et al., 2003). F5H1 is expressed in all tissues, with maximal expression



B

	Roots	Young Stem	Leaves	Internode 6	Internode 1
4695478.2.1	21806	22016	14183	13755	21430
3230260.2.1	-	-	-	8886	-
131555.2.3	-	-	-	8736	-
131555.2.1	-	8362	-	11730	6880
2646712.2.1	-	-	-	8414	-
2969912.2.1	-	-	-	9973	7397
CCR2	10676	-	-	8776	7135
3012873.2.2	22365	16527	10775	37894	11443
Total	54848	46904	24958	108163	54285

Figure 6. Characterization of the CCR gene family in maize. A, Phylogenetic analysis. Classes were defined according to evolutionary distances defined by Phylip 3.5 software. Contig numbers 131555.2.2, 4695478.2.1, 2969912.2.1, 3230260.2.1, and 131555.2.3 annotated as putative CCRs. For CCR2, a contig was not found in the GPI database. B, Gene expression in organs at the four- to five-leaf stage and internodes at different developmental stages. Values indicate the normalized signal hybridization intensity for each gene and (−) signifies a below-background signal intensity of ≤6,000. C, Relative contribution of each gene to total CCR gene expression. R, Roots; L, leaves; YS, young stems, which results from piled-up internodes at the four- to five-leaf stage. IN6, Internode just below the node bearing the ear at silking; IN1, basal internode at silking. Note that equivalent datasets as found in B and C are available for all gene entries in MAIZEWALL.

in developing stems, whereas F5H2 appears to be more closely associated with earlier stages of plant development. In maize, there are two F5H genes: F5H1 was previously described by Puigdomenech et al. (2001) and F5H2 was identified in this study. They exhibited 92% identity between them at the nucleotide level. Maize F5H1 and F5H2 exhibit 60% identity/72% similarity and 51% identity/65% similarity, respectively, with the Arabidopsis F5H1 gene at the protein level. Expression analysis indicated that F5H1 was highly expressed in young stems and with the highest expression in leaves. Interestingly, F5H1 transcripts could not be detected in roots (Table III). On the contrary, F5H2 was predominantly expressed in roots and, to a lesser extent, in other organs with the lowest levels in young stems and IN6 (data not shown).

COMT

COMT was originally thought to be a bifunctional enzyme that sequentially methylated caffeic and 5-hydroxyferulic acids. More recently, it has been shown that COMT acts downstream in monolignol biosynthesis by methylating the aldehyde and alcohol backbones (Osakabe et al., 1999; Parvathi et al., 2001). In maize, a single gene encoding COMT has previously been identified (Collazo et al., 1992). Down-regulation of this gene, both in the *bm3* mutant (Vignols et al., 1995) and in antisense transgenic maize (Piquemal et al., 2002), led to a drastic decrease in lignin content and S-unit lignin. In the GPI database, we detected one contig (no. 2192909.2.3) that annotated COMT. This contig corresponded to the previously described COMT. In agreement with published

expression data (Collazo et al., 1992), this gene was expressed in all organs, with the highest levels in IN6 and the lowest in leaves (Table III).

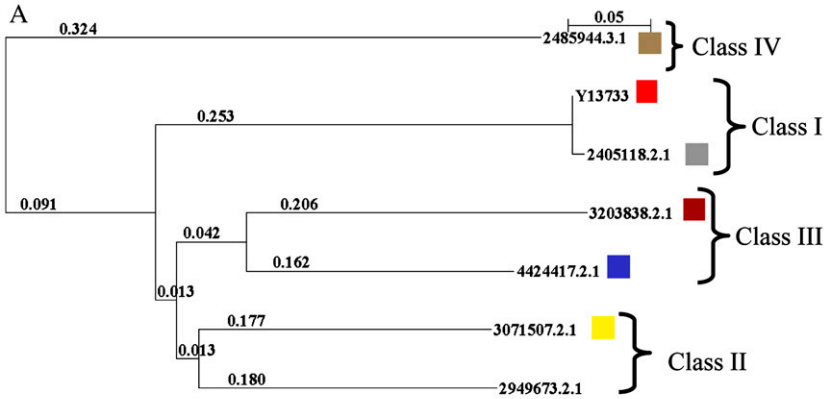
CAD

CAD catalyzes the reduction of *p*-hydroxycinnamaldehydes into their corresponding alcohols and is the last enzyme in monolignol biosynthesis. In the Arabidopsis genome, nine putative CAD genes have been identified (Raes et al., 2003). Whether different CAD genes possess different substrate specificity toward coniferyl and sinapyl alcohol and hence dictate, at least to some extent, lignin composition in planta has been the subject to debate (Li et al., 2001; Sibout et al., 2005). Because this issue has not been addressed in monocots (to our knowledge), we chose to classify all contigs annotating CAD, CAD-like, sinapyl alcohol dehydrogenase (SAD), and SAD-like into one CAD family.

In maize, one CAD cDNA had previously been characterized (Halpin et al., 1998). Its expression was also shown to be severely affected in the *bm1* mutant, resulting in modified lignin content and structure. Beyond the identification of the previously described CAD (Y13733), searching the GPI databases enabled us to identify six other CAD family contigs (Fig. 7A). One contig (no. 2405118.2.1) exhibited 92% identity at the nucleotide level with the original CAD gene (Supplemental Fig. S4E). Sequence identities ranged from 39% to 92% among the different family members. Contig numbers 3071507.2.1 and 2949673.2.1 were most similar to the SAD gene originally characterized in poplar and annotated as such (Li et al., 2001). They exhibit, respectively, 51% and 49% identity at the nucleotide level with the poplar SAD gene.

In young plants, maximal CAD expression was observed in stems (Fig. 7B). The relative contribution of each contig to total CAD expression is quite different at this stage (Fig. 7C). In young stems, whereas five

A



B

	Roots	Young Stem	Leaves	Internode 6	Internode 1
2485944.3.1	-	8729	6550	8210	-
Y13733	13448	10334	6205	30285	9569
2405118.2.1	7346	6635	-	13998	6463
3203838.2.1	-	-	-	9165	-
4424417.2.1	6947	16464	12875	9826	6158
3071507.2.1	-	11048	7150	17398	6445
Total	27741	53210	32780	88882	28635

C

Figure 7. Characterization of the CAD gene family in maize. A, Phylogenetic analysis. Classes were defined according to evolutionary distances defined by Phylip 3.5 software. Note that contig number 2485944.3.1 was annotated putative CAD, contigs numbers 3203838.2.1 and 4424417.2.1 were annotated SAD-like, and contig numbers 3071507.2.1 and 2949673.2.1 were annotated SAD. B, Gene expression in organs at the four- to five-leaf stage and internodes at different developmental stages. The values indicate the signal-normalized hybridization intensity for each gene and (−) signifies a below-background signal intensity of ≤6,000. Note that contig number 2949673.2.1 was not included in CAD expression studies because we were unable to amplify a corresponding GST. C, Relative contribution of each gene to total CAD gene expression. R, Roots; L, leaves; YS, young stems, which results from piled-up internodes at the four- to five-leaf stage. IN6, Internode just below the node bearing the ear at silking; IN1; basal internode at silking. Note that equivalent datasets as found in B and C are available for all gene entries in MAIZEWALL.

of the six spotted genes were expressed at similar levels, in roots only three were expressed with Y13733, the classic maize CAD, accounting for one-half of total CAD gene expression. In leaves, contig number 4424417.2.1 was the predominant CAD gene. In IN6, total CAD transcriptional activity was high and thereafter decreased in IN1 (Fig. 7B). All contigs were expressed in IN6, with Y13733 being the most highly expressed among them. Furthermore, contig number 32038382.1 was only expressed in IN6. In IN1, global CAD expression was low, with Y13733 continuing to be the most highly expressed.

Histochemical Analysis Reveals a Large Diversity of Spatially Regulated, Lignified Cell Types in Maize

To associate global cell wall gene expression with cell wall type and composition, cross-sections of the same organs analyzed by transcriptomics were observed (Fig. 8). Sections were stained with Maüle reagent, which is classically used to distinguish S-unit (red coloration) and G-unit (brown coloration) lignins (Nakano and Meshitsuka, 1992). In primary roots, three to four cell layers of hypodermal cells just beneath the epidermis exhibited strong staining (Fig. 8, A and B). Interestingly, a closer examination of this region revealed that the outermost cell layer had thin walls that stained brown (G units; see Fig. 8B, thick arrow), whereas the three innermost layers had thick walls that stained red-purple (S units). In the central portion of the root (Fig. 8, A and C), the vascular cylinder is delimited from the cortex by the endodermis, which possesses a thickened cell wall containing suberin and lignin known as the Casparian strip (Zeier et al., 1999). At the four- to five-leaf stage, the Casparian strip is weakly stained with Maüle reagent (Fig. 8C). Two types of xylem vessels can be distinguished based on their diameter and location. Xylem vessels of narrow diameter are intercalated with the phloem strands at the periphery and large diameter vessels that alternate with lignified parenchyma cells, forming an inner ring. Both xylem and lignified parenchyma exhibited red-purple coloration, indicating the presence of substantial amounts of S units. Roots are therefore characterized by a wide variety of lignified, S-rich cells with the exception of a single layer of hypodermal cells just below the epidermis.

In leaves, sections were made in the central midrib (Fig. 8, E and G) and in the blade (Fig. 8, D and F). The central midrib contains large and small veins located on the upper side of the midrib (Fig. 8E). Both types of veins are surrounded by sclerenchyma cells, which stain yellow-brown with Maüle reagent (Fig. 8, E and F). In the leaf blade, we can also distinguish small and large veins (Fig. 8D). In this case, only large veins are surrounded by sclerenchyma cells, which also stained yellow-brown with Maüle regent (Fig. 8, D and F). Xylem cells and the cells in between them stained red, indicating the presence of S units (Fig. 8F, large arrow). In conclusion, Maüle staining revealed that leaves are characterized by a large proportion of sclerenchyma cells that probably synthesize mainly H and/or G units, whereas parenchyma cells in association with xylem vessels are rich in S-unit lignin.

At the four- to five-leaf stage, young stems are formed by the piling up of nodes and internodes that will subsequently elongate during development (Fig. 8, H–J). Staining with Maüle revealed very few lignified cells at this stage. Indeed, only the protoxylem elements stained red-purple in both internodes and nodes (Fig. 8, I and J, respectively). These results suggest that the young stem is an excellent indicator of gene expression associated with the onset of lignification in maize. At silking, internode elongation has ceased and a gradient of lignification exists between the young, upper internodes (IN6) and old, basal internodes (IN1) of the plant (Scobbie et al., 1993). To examine this gradient at the cellular level, IN1 and IN6 were histochemically analyzed (Fig. 8, K–M). In IN6, lignifying cells included the cell layer just below the epidermis and sclerenchyma cells surrounding xylem elements that exhibited a centrifugal red-to-pink color gradient (Fig. 8M). On the contrary, in IN1, a large proportion of the total cell surface area was already lignified. Only a few cell layers of parenchyma cells located just inside the peripheral sclerenchyma did not possess a lignified secondary cell wall. Lignified cell types included xylem vessel elements, sclerenchyma just below the epidermis and surrounding the vascular bundles, and a high proportion of lignified parenchyma between vascular bundles (Fig. 8L). All the lignified cells stained red, indicating the presence of S units. Complementary staining techniques (phloroglucinol and Mirande's reagent) were also used to examine lignified tissues in all organs. These results indicated the same spatial distribution and diversity of lignified cell types throughout the maize plant (data not shown). Together, these results indicate that (1) vascular tissue contributes only a minor fraction to total lignin content throughout the maize plant and (2) IN6 constitutes the ideal organ to study highly active constitutive lignification in adult maize plants. These observations are in perfect agreement with transcriptomic data pointing to the importance of phenylpropanoid metabolism genes at this stage of internode development.

Lignin Structure Varies Greatly throughout the Maize Plant

Plant material used for transcriptomics and histochemistry was also subjected to lignin analysis. Lignin structure was investigated by thioacidolysis, which is an analytical degradation method that proceeds by cleavage of labile β-O-4 bonds (Lapierre et al., 1986). The total amount and relative frequency of the H, G, and S units cleaved by thioacidolysis provides an estimate of the amount and composition of these units that are uniquely β-O-4 linked. Comparison of H, G, and S distribution among organs at the four- to five-leaf

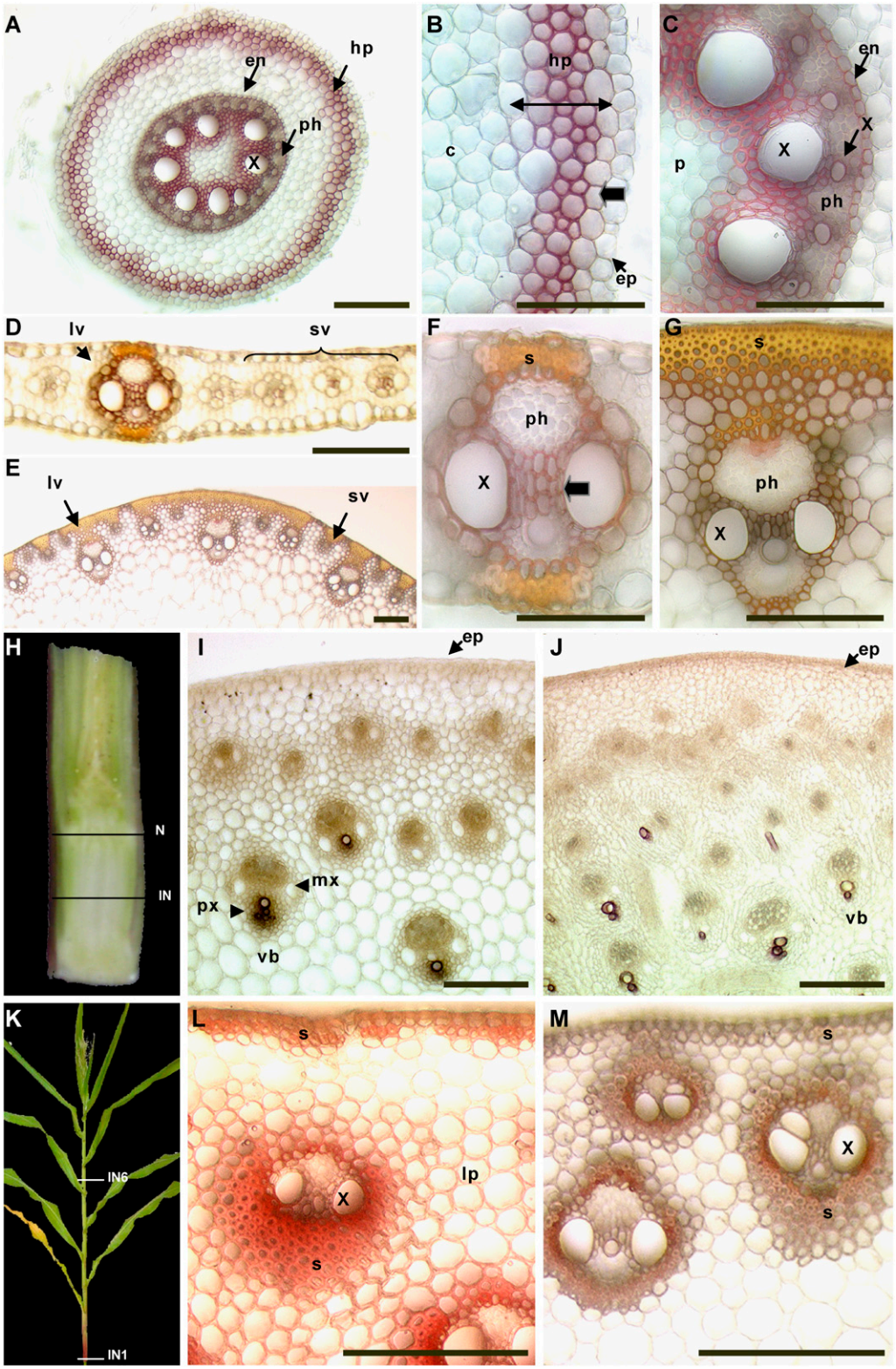


Figure 8. Histochemical localization of lignin in maize organs at the four- to five-leaf stage and internodes at different stages of development at silking. Sections were stained with Maüle reagent. A to C, Transverse sections of primary roots. A, Overall view of a root section. Red-purple staining in the cells of the hypodermal layers and vascular cylinder indicates the presence of S-unit lignin. B, Close-up of hypodermal layers. The three innermost cell layers stained red-purple and the outermost layer just below the epidermis is stained brown, indicating G-unit lignin (large arrow). C, Close-up of the vascular cylinder. The Casparian strip of the endodermis and two rows of xylem vessels embedded in parenchyma cells are all stained red-purple. D to G, Transverse

stage revealed striking differences (Table IV). Root lignin was particularly rich in S units (62.5%), whereas, on the contrary, leaf lignin was particularly rich in G units (72.9%). These results are in good agreement with Maüle histochemical staining. When examining internode development, young stems contain the lowest proportion of S, with slightly more in IN6, followed by IN1. These results suggest that young tissues preferentially accumulate G-unit lignin, whereas S-unit lignin content increases with maturity. This has already been observed in other species (Meyer et al., 1998). Interestingly, H units appear to be more abundant in younger tissues (7.2% in roots, 5.3% in young stems, and 5.4% in IN6) as compared to older tissues (2.2% in IN1). Finally, if one uses thioacidolysis yield (Table IV, H + G + S) as an indicator of lignin content, it may be deduced that IN1 and roots are the most lignified tissues (83 and 53 μmol, respectively), whereas leaves are the least lignified (34 μmol). This is also in agreement with histochemical data. In conclusion, lignin deposition and composition are highly regulated at the spatial level and reflect the extreme diversity of lignified cell types throughout the maize plant.

Table IV. *Lignin composition of maize organs at the four- to five-leaf stage and in internodes at silking*

Each measurement represents the mean of two assays with individual values varying by <3% from the mean.

Samples	H + S + G	S/G	H	G	S
	μmol		*%*	*%*	*%*
Roots	53	2.05	7.2	30.4	62.4
Leaves	14	0.32	3.9	72.9	23.3
Young stem	17	0.40	5.3	67.5	27.3
IN6	34	0.71	5.4	55.4	39.2
IN1	83	1.02	2.2	48.3	49.5

DISCUSSION

MAIZEWALL: A Valuable Tool to Integrate Knowledge of Cell Wall Biosynthesis and Assembly in Maize

At present, our knowledge of cell wall biosynthesis and assembly, especially in monocotyledons, is fragmentary. When taking into consideration the extent of wall polymer diversity, the multitude of ways they assemble in muro, the high degree of wall specificity in different cell types, and the dynamic changes that take place during development within a given cell, it is predicted that several hundreds or even thousands of genes are required for proper, coordinated wall formation. Cell wall-related genomic and proteomic datasets are now available in herbaceous model species, such as Arabidopsis (Zhao et al., 2005; Jamet et al., 2006) and zinnia (Demura et al., 2002; Milioni et al., 2002; Pesquet et al., 2005), and in woody species, including poplar (Hertzberg et al., 2001) and pine (Pavy et al., 2005). Some Web sites are also dedicated to specific protein families, including expansins (http://www.bio.psu.edu/expansins/index.htm), cellulose synthases and cellulose synthase-like genes (http://cellwall.stanford.edu/cesa/index.shtml), and XTHs (http://labs.plantbio.cornell.edu/XTH/overview.htm). In this respect, a large gap exists for monocot species in that genomic inventories specifically devoted to cell walls are lacking. That said, a high-throughput, Fourier transform infrared-based screen has been set up to identify maize plants with modifications in wall assembly or architecture from the UniformMu maize mutant population developed at the University of Florida (http://www.cellwall.genomics.purdue.edu; Bleecker et al., 2004). In this article, we describe the construction of MAIZEWALL, a repertory of 735 genes complete with in-depth bioinformatic analysis and a transcriptional fingerprint of cell wall-related gene expression throughout the maize plant. We consider MAIZEWALL to be a significant leap forward for maize cell wall research while awaiting the full genome sequence in public databases.

The 735 sequences of MAIZEWALL are divided into 174 families based on known gene annotations. As our aim was to subsequently use this database and the corresponding macroarray for a wide range of applications, including mutant analysis, we deliberately chose to widen the scope of entry annotations to include not only primary and secondary cell wall biosynthetic and assembly genes, but also genes involved in closely related metabolism. To provide the most comprehensive and original overview of gene families

Figure 8. (*Continued.*)
sections in leaves. D, Leaf blade. E, Midrib. F, Close-up of a vascular bundle from a large vein in the blade. G, Close-up of a vascular bundle from a large vein in the midrib. In all cases, note the yellow-brown coloration of the sclerenchyma cells above the vascular bundle, indicating the absence of S-unit lignin. H, Longitudinal section through the young stem, resulting from piled-up internodes of plants at the four- to five-leaf stage. Black lines indicate the positions of transverse sections in internodal and nodal regions shown in I and J, respectively. I, Transverse section in the first internode. J, Transverse section in the node. Only walls of the protoxylem are stained red. K, General view of a maize plant at the silking stage grown under greenhouse conditions. White lines indicate the positions of transverse sections of IN1 and IN6 shown in L and M, respectively. L, Transverse sections of IN1 showing red staining of all lignified tissues, including sclerenchyma, parenchyma cells, and xylem. M, Transverse sections in IN6. Note that sclerenchyma cells are very weakly stained and parenchyma cells are not at all. en, Endodermis; ph, phloem; p, pith; ep, epidermal cells; hp, hypodermal cell; c, cortex; s, sclerenchyma; x, xylem; lv, large veins; sv, small veins; vb, vascular bundle; lp, lignified parenchyma; px, protoxylem; mx, metaxylem. Magnification bars: B, C, and F, 100 μm; A, D, E, G, L, and M, 200 μm; I and J, 400 μm.

in secondary wall formation, we also included maize homologs of cDNAs derived from our zinnia TE differentiation library (Pesquet et al., 2005). The sequences were organized into functional categories, including cellulose synthesis, noncellulosic polysaccharide synthesis, general phenylpropanoid, lignin/lignan, transcription factors, etc. This classification was temporary because an actual proof-of-function for any of the MAIZEWALL entries is extremely rare. In our study, as part of the bioinformatic analysis, the most homologous genes in other species have been provided in the database, but these data are based solely on sequence homology and should not be taken as an indication of equivalent gene function. When the maize genome becomes available, it will be necessary to update this search.

Transcriptome-Based Identification of Developmental Marker Genes of Cell Wall Formation in Maize

Global gene expression analysis in different organs and developmental stages of internode development provided us with a fingerprint of the cell wall transcriptome throughout the maize plant. In this way, we identified (1) individual gene family members that are preferentially expressed in a particular organ or developmental stage, and (2) genes that are spatially and temporally coregulated. When considering young plants, the observation that one-third of the genes spotted on the macroarray are not expressed at all is, in itself, of interest. This implies that the transcriptional needs in the early stages of plant development revolve around a restricted set of metabolic functions. When examining the 30 most abundantly expressed genes in all young organs, it is clear that genes involved in polysaccharide biosynthesis and modification genes are predominant. Regarding cellulose synthesis, a cellulose synthase (QBS7b05.xg.2.1), two isoforms of Suc synthase (contig nos. 3748394.2.2 and 131537.2.202), and three endoglucanases (contig nos. 2493751.2.1, 3713002.2.1, and 2922138.2.1) were identified. Among the 14 cellulose synthase genes spotted on the macroarray, only one gene (QBS7b05.xg.2.1) exhibited consistently high levels of expression, not only throughout young plants but also during later stages of internode development. The expression patterns of 12 cellulose synthase genes (ZmCesA1–12) in maize have been previously reported (Holland et al., 2000; Appenzeller et al., 2004). ZmCesA1 to 9 were considered more closely associated with cellulose synthesis in primary walls, whereas ZmCesA10 to 12 were more closely associated with secondary wall cellulose synthesis. QBS7b05.xg.2.1 shares 95% identity with ZmCesA12. The most homologous Arabidopsis gene is *irx3*, which has been clearly shown to be associated with secondary wall cellulose biosynthesis (Taylor et al., 1999). Detailed functional analysis of QBS7b05.x.g.2.1 in maize would enable us to determine its role in either primary or secondary wall cellulose metabolism. Similarly, two of the four spotted contigs annotated UDP-Glc dehydrogenase were among the most highly expressed genes in all young tissues (but not in IN6 or IN1). UDP-Glc dehydrogenase is a key enzyme that oxidizes UDP-Glc to UDP-GlcUA and thus directs carbohydrates irreversibly into the cell wall-specific pool of nucleotide sugars. These transcriptomic data allowed us to identify major actors of cell wall metabolism in young maize tissues, some expressed organ preferentially and others expressed ubiquitously.

A completely different transcriptome fingerprint was obtained when examining the 30 most highly and preferentially expressed genes at midstage internode development (IN6). Clearly, the most highly abundant and/or preferentially expressed transcripts in IN6 include genes in secondary cell wall synthesis and, more particularly, certain gene family members of the lignin biosynthetic pathway (three PALs, one C4H, one 4CL, two CCoAOMTs, one CCR, one COMT, one F5H1, and one CAD). These transcriptomic data are in perfect agreement with cytological observations indicating that IN6 is clearly at the onset of lignification. Moreover, these results suggest highly coordinated transcriptional coregulation among many genes along the monolignol biosynthetic pathway. It is interesting to note that Myb and monopteros transcription factors are also preferentially expressed in IN6, making them excellent candidates for transcriptional regulation of lignin biosynthesis. Myb factors have already been shown to play a role in the coordinated regulation of lignin genes in several different plant species (Karpinska et al., 2004; Goicoechea et al., 2005).

Toward a Division of Labor among Family Members of Lignin Biosynthetic Genes in Different Organs

Until now, the known actors involved in dicot lignin biosynthesis have not been studied in any detail in maize, with the exception of a few isolated gene expression studies for COMT (Capellades et al., 1996), CCR (Pichon et al., 1998), and CAD (Halpin et al., 1998). Allelic variations have also been evaluated for two CCoAOMT and COMT genes in 34 lines of maize selected for their varying digestibility (Guillet-Claude et al., 2004). Herein, we combine cytological, chemical, and transcriptomic approaches as a first step in determining the gene family member for each of the 10 enzymatic steps of the monolignol biosynthetic pathway that is most likely involved in lignin formation in different organs and stages of internode development. As a general rule, monolignol biosynthetic enzymes that are encoded by multigene families in Arabidopsis are also encoded by multigene families in maize, although the numbers may vary. A similar observation has already been made between rice and Arabidopsis (Yokoyama and Nishitani, 2004). Although these differences in gene number certainly exist among species, the predicted number of genes for a given function also depends on the bioinformatic parameters used for gene annotation in the different studies. That said, we did find some essential differences in gene number

between Arabidopsis and maize. First, C4H and HCT are encoded by single genes in Arabidopsis, whereas we identified two genes for each of these functions in maize. Second, in Arabidopsis, C3H is encoded by three genes, whereas only one was identified in maize. It is possible that other maize C3Hs may be identified when the complete genome sequence becomes available.

In Supplemental Figure S5, we have compiled gene expression data for each of the 10 gene families (based on Figures 3–7 and Table III) to propose the most likely routes of monolignol biosynthesis in young organs and during internode development in maize. This scheme is based exclusively on relative gene expression levels for each family, and, for this reason, we cannot exclude that the most highly expressed genes may be involved in the synthesis of other phenylpropanoid metabolites and not necessarily monolignols.

Is There a Sclerenchyma-Specific Route for G-Unit Lignin Synthesis in Leaves?

Lignin analysis of maize plants at the four- to five-leaf stage indicated that leaves were characterized by a high proportion of G units. Similar results have already been reported for leaves at the flowering stage (Piquemal et al., 2002). Our cytological observations revealed that leaf veins are surrounded by patches of heavily lignified sclerenchyma cells that account for a large majority of lignified cells in leaves. These cells stain yellow-brown with Maüle, thereby explaining the high G-unit lignin content in leaves. To our knowledge, G-rich sclerenchyma fibers have not been described in higher plants. In dicots, it is commonly accepted that xylem vessels contain G units, whereas fibers and parenchyma contain both G- and S-unit lignin, with the latter predominating in fibers (Donaldson, 2001). These results suggest that a novel genetic program leading to G units in sclerenchyma fiber cells may be functional in maize leaves.

When analyzing gene expression data, we noted that some members of the multigene families of the monolignol pathway exhibited proportionally high levels of expression in the leaves. In the case of PAL, among the five contigs, three were expressed in leaves, one of which (contig no. 3858663.2.1) contributed to the large majority of total PAL transcriptional activity in leaves. This class II maize PAL is most homologous to Arabidopsis PAL2. In Arabidopsis, PAL2 is expressed in all organs, although most abundantly in roots and stems (Raes et al., 2003). Based on gene expression data, Arabidopsis PAL2, along with PAL1, was considered to be most likely involved in constitutive lignin biosynthesis in Arabidopsis. Detailed sequence analysis of the promoter region of PAL2 indicated the presence of an AC box. Interestingly, the authors hypothesize that this AC box may be a potential signpost of genes involved in G-unit lignin synthesis (Raes et al., 2003). Continuing along the pathway, among the 4CL genes in maize, one of them, 4CL, is the most highly expressed in leaves. The maize 4CL gene is most homologous to the Arabidopsis 4CL1 gene, which, along with Arabidopsis 4CL2, is considered to be involved in constitutive lignification. Again, the presence of an AC box in the promoter region in 4CL1 from Arabidopsis was indicative of G-unit synthesis. By combining cytological, biochemical, and transcriptome data, it is tempting to speculate that the maize PAL (contig no. 3858663.2.1) and 4CL channel phenylpropanoid intermediates into a G-rich sclerenchyma pathway in maize leaves. However, we cannot exclude that these genes function in a distinct branch of phenylpropanoid metabolism in leaves. Promoter analysis of these and laser microdissection techniques coupled to metabolic profiling would be particularly useful to confirm the hypothesis that there is a G-unit monolignol pathway in the sclerenchyma cells of maize leaves.

Finally, transcript profiling of G-rich leaves yielded some unexpected results and raises new questions. First, among CAD family members, contig number 4424417.2.1 is the most highly expressed in leaves. This gene was originally annotated as SAD like. Our expression data are in apparent contradiction with the idea that this branch of the CAD family phylogenetic tree is specifically dedicated to S-unit lignin synthesis. Second, F5H1, again normally associated with S-unit lignin synthesis, is among the 30 most highly expressed genes in leaves. This observation obliges us to reexamine the role of this F5H gene in maize leaves. In any case, all of these genes must be subjected to in-depth biochemical and molecular characterization to precisely determine their role in planta.

Is There a Root-Specific Pathway to Synthesize S-Unit Lignin?

A question that should be addressed in detail is whether lignin biosynthesis occurs via the same suite of enzymatic steps in all organs and at all developmental stages during plant life. This is probably due to the fact that most biochemical and molecular studies on lignification focus primarily on stems of model, herbaceous plants or wood. Some clues may be obtained from the recent characterization of the C3H1 mutant in Arabidopsis (Abdulrazzak et al., 2006). As expected, the aerial portions of the mutant had lower lignin content and were almost exclusively composed of H units, with only trace amounts of G and S units. More surprisingly, in roots, lignin contained both G and S units in significant amounts. These results suggest that the hydroxylation step leading to G and S synthesis must occur via a C3H-independent pathway in Arabidopsis roots. In keeping with this hypothesis, the unique C3H gene identified in maize is expressed at barely above background levels in roots and it would be difficult to imagine that this C3H enzyme could, in itself, account for the total flux of phenylpropanoid metabolites into G and S units in roots. However, the fact that a relatively high level of

H-unit lignin was measured in roots, concomitantly with low C3H expression levels, on the contrary, may indeed suggest that C3H may be involved in controlling the channeling of monolignols from the H- to G- and S-unit pathways. It is interesting to note that overall C3H expression is relatively low not only in roots but also, to a lesser extent, throughout the maize plant. Inefficient C3H-mediated channeling may explain, at least in part, the accumulation of H-unit lignin in maize (and in monocots in general) in comparison to dicot species.

In maize, lignin analysis of roots from plants at the four- to five-leaf stage indicated an extremely high proportion of S-unit lignin as compared to the other organs studied. Maize roots therefore provide a unique opportunity to probe S-unit synthesis in maize. Several studies carried out in dicots underline the importance of F5H1 in the synthesis of S units in Arabidopsis (Meyer et al., 1998; Humphreys et al., 1999; Ruegger et al., 1999), tobacco, and poplar (Franke et al., 2000). Unexpectedly, transcriptome profiling of S-unit-rich roots of maize revealed that F5H1, the most likely functional homolog of F5H1 from Arabidopsis based on sequence analysis, was not expressed at all in roots. However, RT-PCR analysis did indicate that a distinct F5H, F5H2, was expressed, but only slightly, in maize roots (data not shown). These data suggest that the hydroxylation of coniferaldehyde and/or coniferyl alcohol for S-unit synthesis may occur either via F5H2 or perhaps by a totally different pathway in maize roots. Interestingly, in Arabidopsis, neither of two F5H genes is even moderately expressed in roots compared to the other organs (Raes et al., 2003), raising the question as to whether, even in Arabidopsis, F5H is involved in S-unit synthesis in roots. It would be interesting to determine whether lignin composition of the F5H mutant (*fah1*) is altered in roots. Taken together, it is not clear whether roots from all species use a common hydroxylation/methylation pathway for G-unit and S-unit lignin synthesis that is different from the aerial portions or whether roots use alternative pathways for G-unit and S-unit lignin synthesis in a species-specific manner. In any case, based on the relatively high proportion of H units typically found in monocot versus dicot lignin, it is reasonable to assume that some specificity in terms of the genes and regulatory mechanisms of hydroxylation/methoxylation pathways exist in monocots.

In the search for clues to explain high S-unit lignin content in roots, we closely examined the CAD family transcriptome because it is still unclear as to whether CAD/SAD isoforms orient G- and S-unit synthesis, respectively. Li et al. (2001) originally identified an enzyme in poplar with high sequence homology to the traditional CAD gene, but with higher substrate specificity for sinapaldehyde than for coniferaldehyde. The authors called this enzyme SAD and proposed that SAD was required for S-unit biosynthesis, whereas CADs were required for G-unit lignin biosynthesis. However, more recently, Sibout et al. (2005) reported that, in Arabidopsis, CAD genes were sufficient to produce S-unit lignin without the requirement for SAD genes. Our results indicate that the classic CAD (Y13733) is by far the predominantly expressed CAD in roots. This finding further supports the hypothesis that CAD is sufficient to produce both S and G units, even in maize.

Above and beyond the benefits for maize lignin research, MAIZEWALL is unique in that it contains an in-depth bioinformatic analysis and expression data on a wide range of cell wall-related families in a monocot species within a single interface. It provides the groundwork to significantly enhance our basic knowledge of cell walls in maize and can be of immediate use for a wide range of agronomic and industrial pursuits ranging from forage digestibility to bioethanol production (Boudet et al., 2003).

MATERIALS AND METHODS

Plant Material

The well-characterized F2 maize (*Zea mays*) line was used in this study. Plants were grown in pots with a mixture of sand and compost and irrigated with nutritive solution in the greenhouse during the spring of 2004 in Lusignan, France. Plants were harvested when they had four to five expanded leaves or at silking just before pollen shed. At the four- to five-leaf stage, roots, piled-up internodes (referred to as young stems throughout the text), and leaves were harvested. At the silking stage, the basal internode (IN1) and the internode just below the node bearing the ear (IN6) were sampled. Nodes and leaf sheaths were eliminated. For transcriptome analysis, all samples were frozen in liquid nitrogen and conserved at −80°C. Nine plants were divided into three pools of three plants each for transcriptome analysis and all plants were pooled for lignin analysis.

Database Construction

The MAIZEWALL database was constructed using two gene sources. The first was based on a list of nearly 100 keywords from existing knowledge of primary and secondary cell wall biosynthesis and assembly, and vascular patterning was established. These keywords fell under different categories: cellulose synthesis, noncellulose polysaccharide synthesis, nucleotide sugar synthesis, conversion and transport, cell wall proteins, general phenylpropanoid, lignin/lignan, etc. Keywords were used to search the PubMed, Nucleotide, and Protein resources of the National Center for Biotechnology Information (NCBI) databases to retrieve all available information related to each keyword (literature references, nucleotide and protein sequences, respectively). Searches were first performed using keyword ''AND Zea'' (to obtain a maize sequence when available). If a maize sequence was unavailable, keyword ''AND plant'' was used to obtain a plant sequence that was subsequently used (see below) as bait to retrieve unannotated sequences in maize databases. The second source of genes corresponded to maize genes exhibiting homology with ESTs from a zinnia (*Zinnia elegans*) subtractive library enriched in genes expressed during secondary wall formation of in vitro TEs (Pesquet et al., 2005). Briefly, 232 NR ESTs were generated by suppressive subtractive hybridization carried out on in vitro differentiating TEs of zinnia at several selected time points corresponding to presecondary cellulose thickening, prelignification, and early cell death.

All of the protein and nucleotide sequences from the two above-mentioned strategies were used to search the most homologous maize sequences in the MaizeGénoplanteContigsVersion2 database (GPI2). The Génoplante database includes mRNAs retrieved from NCBI and ESTs from the Génoplante program, dbEST, and Stanford resources (Samson et al., 2003). A global clustering of sequences was performed to produce gene-specific contigs. At the time of this writing, GPI2 contained nearly 60,000 contigs.

An overall scheme for the construction of MAIZEWALL can be found in Figure 1. MAIZEWALL was written in PHP language (http://www.php.net),

runs on an Apache2 server, and interacts with MySQL (http://www.mysql.com) to save data in the form of tables and make queries related to the data. PHP script uses some applications on the GPI secure interface, such as BLAST2 (Altschul et al., 1997), ClustalW (Thompson et al., 1994), Dialign2 (Morgenstern, 1999), Multalin (Corpet, 1988), eprimer3 (Rozen and Skaletsky, 2000), SWALL (SWISS-PROT, TrEMBL, and new databases), NR protein database, Génoplante contig databases, and some EMBOSS package programs (revseq and transeq; Rice et al., 2000). Several programs were also applied to Arabidopsis (*Arabidopsis thaliana*) protein sequences that correspond to homologs of maize-selected contigs to search protein domains, signal peptide position, transmembrane domains, and subcellular targeting sequences. They include Aramemnon (Schwacke et al., 2003), MIPS (Schoof et al., 2002), PSORT (Nakai et Horton, 1999), PROSITE (Sigrist et al., 2002), SMART (Schultz et al., 2000; Finn et al., 2006), ProDom (Corpet et al., 1998), SignalP (Bendtsen et al., 2004), TMHMM (Krogh et al., 2001), TargetP (Emanuelsson et al., 2000), and Predotar (Small et al., 2004).

For zinnia sequences, BLASTn and tBLASTx searches were performed directly on GPI2. Maize sequences with an e value of e^{-05} from zinnia sequences and an e value of e^{-20} from a keywords search that annotated with the expected putative function against SWALL (SWISS-PROT, TrEMBL, and new databases) and NR databases were included in MAIZEWALL. In total, 735 contigs met these criteria. All of the MAIZEWALL entries were used to retrieve the closest available sequence in other plant species, including rice (*Oryza sativa*), pine (*Pinus sylvestris*), poplar (*Populus tremuloides*), Arabidopsis, wheat (*Triticum aestivum*), barley (*Hordeum vulgare*), sorghum (*Sorghum bicolor*), and Medicago.

To construct the macroarray, we designed specific primer pairs to amplify gene-specific PCR fragments for each gene. Toward this end, contigs that annotated with the same putative function (and hence with a high degree of homology) were multialigned using ClustalW, Dialign2, and Multalin programs. The 3′-UTRs were defined and specific primer pairs designed using the eprimer3 program (20 bp, Tm $\approx$ 58°C–60°C) to amplify GSTs of 150 to 250 bp in length. GST specificity was confirmed by BLAST against GPI2 to ensure that no hits were detected except for the contig of origin.

Macroarray Construction

To design the macroarray that would guarantee maximal signal specificity and sensitivity, a pilot experiment was carried out on a set of three *O*-methyltransferase genes selected for their known expression profiles. The experiment was designed to optimize the following parameters: (1) quantity of cDNA spotted on the membrane; (2) source for probe synthesis (total RNA versus polyA RNA); (3) probe type (reverse-transcribed cDNA versus random priming); (4) length of GSTs spotted; and (5) relative proportion of 3′-UTR and coding sequence to ensure hybridization specificity within a multigene family. The pilot macroarray contained COMT (M73235), CCoAOMT1 (AJ242980), and CCoAOMT2 (AJ242981). The results of this pilot array enabled us to define technical parameters for the cell wall macroarray as follows: spotted PCR products corresponded to the 3′-UTR of 150 to 250 bp in length at a concentration of 0.25 μg/μL (100 nL/spot); the cDNA probe was generated from reverse transcription of total RNA.

Six hundred fifty-one GSTs were spotted on the macroarray. The GSTs were amplified by PCR in 96-well PCR plates in a total volume of 50 μL/well with 25 μL of 2× Master Mix PCR (Promega), 4 μL of forward and reverse specific primers for each GST (10 μM each), 20 μL of ultrapure water, and 1 μL of the recombinant bacterial culture containing the corresponding plasmid. To obtain PCR products of genes for which a viable bacterial culture was not available, RT-PCR was performed on a pool of roots, young stems, and leaves of the F2 line using standard procedures (Promega). All primers were synthesized by MWG-BIOTECH.

PCR products were transferred to Eppendorf tubes, precipitated in sodium acetate (0.3 M) and isopropanol overnight at −20°C, and centrifuged for 30 min at 12,000 rpm. Pellets were then rinsed twice in 70% ethanol, dried, and resuspended in 20 μL of Tris-EDTA buffer (pH 8.0). PCR products were checked for a single band of the expected size and quantified at 260 nm with a Biophotometer (Eppendorf) and by electrophoresis on 2% agarose gels. They were diluted to a final concentration of 0.5 μg/μL. PCR products were then denatured in 50% DMSO and transferred to 384-well plates. Controls were added in separate 384-well plates. One plate included 384 Tris-EDTA, pH 8.0 (blank background control), and another plate with 30 NPT II fragments (a positive hybridization control), 20 pBluescript plasmids (unspecific hybridization control), and 18 ubiquitin fragments (positive control) prepared as indicated above (Pesquet et al., 2005). All fragments were then spotted onto a 20- × 20-cm Nytran SuPerCharge nylon membrane (Schleicher and Schuell) using a BioGrid spotting robot (BioRobotics) in a 4 × 4 grid organization with each spotted in duplicate within a grid. Each amplified GST was also spotted in two different grids, which means that each GST was spotted four times on each macroarray. All macroarrays were UV cross-linked at 1,200 × 100 μJ/cm^2 prior to use.

RNA Extraction and Purification

Total RNA was isolated from 5 g of each sample according to the method described by Ragueh et al. (1989). After grinding in liquid nitrogen, the material was resuspended in extraction buffer (200 mM Tris, pH 7.0, 20 mM EDTA, 100 mM NaCl, 1% SDS) and extracted twice with phenol:chloroform:isoamylalcohol (25:24:1). Total RNA was precipitated with sodium acetate (0.3 M) with isopropanol. The aqueous phase was separated by centrifugation (20 min, 12,000 rpm) and the pellet resuspended in nuclease-free water. Finally, RNA was extracted with phenol:chloroform:isoamylalcohol (25:24:1) and twice with chloroform:isoamylalcohol (24:1). After extraction, LiCl (3 M) was added to the aqueous phase and left overnight at 4°C. RNA was precipitated by centrifugation (30 min at 12,000 rpm) and the resulting pellet rinsed with ethanol (100%) and resuspended in sterile, distilled water. Total RNA was subjected to DNA digestion with 5 units of RNase-free DNase I (Promega) for 1 h at 37°C. RNA content was measured at 260 mm with a Biophotometer (Eppendorf) and visualized by electrophoresis on 1.5% agarose gels. RNA concentration was then adjusted to 1 μg/μL for further use. To obtain polyA RNA, Dynabeads oligo(dT)$_{25}$ (Dynal Biotech ASA) were used according to the Dynal Biotech protocol (http://www.invitrogen.com).

cDNA Probe Synthesis and Hybridization

cDNA probe synthesis and membrane hybridization were performed according to Pesquet et al. (2005) with two slight modifications. First, probes were synthesized from 10 μg of total RNA for each sample. Second, after hybridization at 65°C, membranes were washed more stringently at the same temperature: twice for 15 min in 3× SSC/0.5% SDS, once for 15 min in 1× SSC/0.5% SDS, once for 15 min in 0.5× SSC/0.5% SDS, and once for 15 min in 0.1× SSC/0.5% SDS. Membranes were wrapped in cellophane and placed in a PhosphorImager cassette (Molecular Dynamics, Amersham-Pharmacia) for 72 h and scanned at 50 μm/pixel by a Storm 820 scanner (Amersham-Pharmacia).

Statistical Analysis of Gene Expression

Data analysis was performed according to Pesquet et al. (2005). Macroarray gridding and gene expression levels were measured with ImageQuant 5.0 software (Molecular Dynamics, Amersham-Pharmacia) using 4 × 4 grids. Grids were adjusted manually and tested with regard to the distance of the maximal intensity value from the centroid of the measured area in x and y axis. Expression data for all gene sequences were analyzed using the Microsoft Excel program. Normalization between samples was established using the linear slope defined by the blank background, unspecific hybridization, and positive hybridization controls (Tris-EDTA, pH 8, pBluescript, ubiquitin, and NPT II, respectively) in the different samples (Supplemental Fig. S1). The slope defined by the controls indicated a linear correlation factor of R^2 close to 1.

Preliminary analyses were first performed to assess the inner-membrane variation of duplicate spots for each gene on the same membrane and the reproducibility of spot intensity ratios (expressed as a $\log_{10}$ expression ratio [LR]) resulting from hybridizations of independent membranes using independent probes derived from independent biological samples. The data obtained were highly reproducible within a given membrane and, even more importantly, between two independent membranes (Supplemental Fig. S2). In the experiment illustrated in Supplemental Figure S2, 96% of the values fell within ±0.176 LR of the mean (equivalent to a 1.5-fold difference) and 99% within ±0.3 LR (equivalent to a 2-fold difference). Macroarray reproducibility was further analyzed by comparing spot intensity values from three independent hybridizations performed on three independent membranes with probes from three independent samples. Linear coefficients of determination between experiments were calculated, thereby defining high reproducibility between independent hybridizations with more than 95% of the values confined within a 2-fold limit (Supplemental Fig. S3). The average signal value from triplicate hybridizations and SD were calculated for each gene

(Supplemental Table S4). Genes whose SDs exceed their average signal values were eliminated from the Microsoft Excel spreadsheet gene list. Taking into consideration background signal intensities, a gene was considered expressed when its intensity was greater than 6,000 and, in order for a gene to be considered as differentially expressed between two hybridizations, the difference in spot intensities had to be greater than a 2-fold significance threshold.

Phylogenetic Analysis of Lignification Genes

For the construction of phylogenetic trees, contig sequences were aligned with the ClustalW 1.83 program (Thompson et al., 1994) and the relation between them was then investigated with the Phylip 3.5 package (http://evolution.genetics.washington.edu/phylip.html). Sequence alignments were performed with the Needle program of EMBOSS software suite (Rice et al., 2000).

Lignin Histochemical Staining

Leaf, stem, and root sections were cut with a vibratome. Maüle reactions were performed according to standard protocols (Nakano and Meshitsuka, 1992). Sections were observed using an inverted microscope (Leitz DMIRBE). Images were registered using a CCD camera (Color Coolview; Photonic Science) and treated by image analysis (Image Pro-Plus; Media Cybernetics).

Lignin Analysis

Leaves, roots, and young stems of four- to five-leaf-stage plants and IN1 and IN6 of flowering plants were lyophilized at harvest and ground to a fine powder. Lignin monomeric composition was determined by thioacidolysis followed by gas chromatography (GC)-mass spectrometry (MS) of lignin-derived monomer trimethylsilylated (TMS) derivatives (Lapierre et al., 1986).

Thioacidolysis reagent contained 2.5 mL of BF3 etherate and 10 mL of ethanethiol, adjusted to a 100-mL volume with dioxane. Each dried and ground sample fragment (dry weight ranging from 10–30 mg) was added to 10 mL of reagent and 1 mL of a docosane solution (0.1 mg/mL in CH_2Cl_2 as GC internal standard) in a glass tube closed with a Teflon-lined screw cap. Thioacidolysis was performed at 100°C for 4 h. The cooled reaction mixture was diluted with 30 mL of water and its pH was adjusted to 3 to 4 with aqueous $NaHCO_3$. The reaction mixture was extracted with CH_2Cl_2 (3 × 30 mL). Combined organic extracts were dried over Na_2SO_4 and then evaporated under reduced pressure at 40°C. The final residue was resolubilized in 1 mL of CH_2Cl_2 before silylation and GC-MS analysis as previously described (Lapierre, 1993). The quantitative determination of the main H-, G-, and S-unit lignin-derived monomers, analyzed as their TMS derivatives, was carried out from specific ion chromatograms reconstructed at *m/z* 239 for the H monomers, 269 for G monomers, and 299 for S monomers after an appropriate calibration relative to the docosane internal standard. These acids were estimated from specific ion chromatograms reconstructed at *m/z* 338 (FE) and 308 (PC), which respectively corresponds to the molecular ions of their TMS derivatives.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Normalization between independent experiments.

Supplemental Figure S2. Scatter plot of duplicate spots from independent hybridizations.

Supplemental Figure S3. Scatter plot of normalized spots of independent experiments.

Supplemental Figure S4. Nucleic acid identities of lignin genes.

Supplemental Figure S5. Hypothetical routes of monolignol biosynthesis in maize.

Supplemental Table S1. Cell wall gene catalog of MAIZEWALL.

Supplemental Table S2. Most highly expressed genes in young plants.

Supplemental Table S3. Most highly expressed genes in stems.

Supplemental Table S4. Statistical analysis of macroarray data.

ACKNOWLEDGMENTS

We sincerely thank Edouard Pesquet for his invaluable help in the initial phases of database construction and macroarray analysis. We are also grateful to Odile Barbier for technical support, Brigitte Pollet for thioacidolysis analysis, and Dominique Denoue for growing maize plants. We thank Mickaël Bosio and Jean-Pierre Martinant (Biogemma, Clermont-Ferrand) for fruitful discussions and David Rouquié (Rhobio, Evry) for providing some of the EST sequences. Laurent Decousset (Biogemma, Clermont-Ferrand) provided us with all of the plasmids. For macroarray spotting, we acknowledge Nathalie Ladouce (Génopole, Toulouse). And, finally, we would like to thank Alain Michel Boudet for a critical review of the manuscript, and Christophe Pineau and Philippe Ranocha for helping with database design.

Received July 7, 2006; accepted November 3, 2006; published November 10, 2006.

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