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Genetics and Genomics of Lignification in Grass Cell Walls Based on Maize as Model Species

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ABSTRACT

Grass lignins are made of guaiacyl and syringyl units together with minor amounts of *p*-hydroxyphenyl units. The specific association of *p*-coumaric (pCA) and ferulic (FA) acids to grass lignins has suspected important consequences on wall properties which are however, poorly understood. Genetic variation for lignin content, lignin structure, and *p*-coumaric and ferulic acid contents has been shown in normal maize, after an earlier description in brown-midrib (*bm*) mutants. QTL analyses for lignin-related traits have established that nearly 40 genomic regions are involved in maize variation of lignin content. For most of these locations, no candidate genes have been validated, or have been still defined. Whereas all steps of lignin biosynthesis have been presumably identified, little is known about the number of gene members encoding each enzymatic step and the role of each member in organ, stage and/or tissue specificity. Moreover, even if the lignin pathway has often been displayed as a metabolic grid, available results, especially in maize *bm* and genetically engineered plants, suggest that the hydroxylation/methylation steps at the aromatic C-3 position have a key role in controlling the flux to lignins. Recent studies of cell wall-related gene expression in young and silking maize plants also illustrated an unexpected diversity of genes with differential expression profiles, especially in *bm* mutants. Breeding maize and other grasses for phenolic structures more suitable for animal nutrition or energy production could now be considered a realistic goal by integrating new genomic-based knowledge on maize lignification.

Keywords: expression study, ferulic acid, lignin, *p*-coumaric acid, QTL
Abbreviations: **ALDH**, aldehyde dehydrogenase; **COMT**, caffeic acid *O*-methyltransferase; **CCoAOMT**, caffeoyl-CoA *O*-methyltransferase; **CHI**, chalcone flavonone isomerase; **CCR**, cinnamoyl-CoA reductase; **CAD**, cinnamyl alcohol dehydrogenase; **C3H**, coumarate 3-hydroxylase; **C4H**, cinnamate 4-hydroxylase; **4CL**, 4-coumarate:coenzyme A ligase; **pCA**, *p*-coumaric acid; **F5H**, ferulate 5-hydroxylase; **FA**, ferulic acid; **G**, guaiacyl; **5-OH-G**, 5-hydroxyguaiacyl; **H**, *p*-hydroxyphenyl; **HCT**, hydroxycinnamoyl-CoA transferase; **OMT**, *O*-methyl transferase; **PAL**, phenylalanine ammonia lyase; **SAMS**, *S*-adenosyl-methionine synthetase; **SAD**, sinapyl alcohol dehydrogenase; **S**, syringyl

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Invited Review

INTRODUCTION

Nearly one third of the earth's vegetative cover is dominated by grass (or *Poaceae*, monocotyledon) ecosystems (Jacobs *et al*. 1999). Grasslands and savannas, dominated by *Poaceae* and covering about 20% of earth's landscape, are a major source of nutrients for wild and domesticated herbivores. *Poaceae* cereals dominate cultivated cropland and their grains supply most of the dietary energy needs of people. Grains of cereals are also a major energy resource for many classes of livestock, while straw of small grain cereals, including rice, is also used as low-cost forages of lower feeding value. To date, both grains of cereals and their straw or stover are considered for bio-fuel production.

Lignification and the development of a vascular system have allowed plants to leave aquatic habitats and to acquire erect growth, even if the earliest vascular thickenings were unlignified and if the cell wall lignification appeared in the outer cortex of Devonian land plants, 400 million years (mya) ago, probably as a defense mechanism against pathogens (Boyce *et al*. 2003). Lignins are important for the structural integrity of stems and they contribute with cellulose to the mechanical properties of these stems. Lignins also impart hydrophobicity to vascular elements allowing water and nutriment transportation. However, vascular organizations are deeply different when comparing monocotyledons and dicotyledonous plants. The divergence between monocotyledons and dicotyledonous plants is very ancient and dated to 200 mya, while the first grasses, which were plants of forest margins, appeared nearly 70 mya, in late Cretaceous or early Tertiary (Wolfe *et al*. 1989; Herendeen and Grane 1995; Jacobs *et al*. 1999; Kellogg 2001). In contrast to dicotyledonous plants, the vascular system of monocotyledons is characterized by the absence of bifacial cambium and secondary growth. In monocotyledons, lignification proceeds from an intercalary meristem in each internode, with vascular bundles scattered, penetrating radially and present in medulla and cortex. The monocotyledon vascular system has been considered as having no homology with the dicotyledons vascular system, which could not be considered as ancestral (Terashima and Fukushima 1993; Tomlinson 1995). These vascular specific traits in grasses can make it difficult to directly transfer data and knowledge related with tissue organization and patterning from dicotyledons and gymnosperms to grasses. However, most of all results obtained in genetics and genomics of lignification illustrated a large commonality of cell wall carbohydrate and phenolic biosynthesis in plants. Based on fossil plants studies, the evolution of lignified tracheids and vascular tissues might indeed involve the expression a pre-existing poly-phenolic pathway and its targeted deposition in different cell types (Boyce *et al*. 2003).

Grasses mostly comprise two clades, with additionally three basal lineages (*Anomochlooideae*, *Pharoideae* and *Puelioideae*) which are actually represented by only 25 species (GPWG 2001; Kellogg 2001). The oldest diverging clade (BEP) is comprised of *Bambusoideae*, *Ehrhartoideae* (about 105 species, including rice) and *Pooideae* (about 3300 species, including small grain cereal crops and temperate C3 forage grasses). The earlier diverging clade (PAC CAD) is comprised of *Panicoideae* (about 3250 species), which are thought to have a Gondwanian origin (Clayton 1981; Arriaga 2000), and five others families (*Arundinoideae*, *Centhothecoideae*, *Chloridoideae*, *Aristidoideae* and *Danthonioideae*). The Maydeae tribe is, among *Panicoideae*, the most recently diverging one. Rice, with 12 haploid chromosomes, is an ancestral form and reflects the genome of the ancestral grass from which more elaborated grasses evolved. Divergence between rice and maize progenitors occurred 60 to 80 mya ago, rapidly after grass emergence and earlier than divergence between rice and *Pooideae* (Bennetzen and Freeling 1997; Gaut 2002). However, despite the actual large diversity in species and growth habit, plants of the grass family have well conserved genomes (Devos 2005), and are very likely of monophyletic origin. Moreover, within grasses, *Pooideae* and *Panicoideae* are both monophyletic (Kellogg and Linder 1995; Linder and Kellogg 1995). Only 19 linkage blocks (large chromosome segments or whole rice chromosomes) are needed to reconstruct all the grass chromosomes compared so far (Moore *et al*. 1995). Maize is now considered as a segmental allotetraploid originating from plants having 8 haploid chromosomes (Wilson *et al*. 1999), with the maize genome estimated to be equivalent to 1.5 rice genomes. Moreover, it has been established that micro-colinearity is also conserved between grasses in most of cases, even if number of exceptions were also demonstrated (Bennetzen and Ramakrishna 2002; Feuillet and Keller 2002). The macro- and partial micro-colinearity of grass genomes is thus an inescapable tool in the search of genes involved in pathways such as those of lignification and/or cell wall biosynthesis.

Most studies related to histology, biochemistry, genetics and genomics of lignification have been conducted in woody dicotyledonous or gymnosperm species. This focus is due to the major interest in reducing the cost and environmental impact of removing lignin from woody plants during pulping for paper production. Plant lignification has also been investigated, at a lower extent, in forage plants (forage grasses, alfalfa) due to a negative correlation between lignin content and feeding value. Lignins are likely the only cell wall component resistant to bacterial and fungal degradation in the gut or in industrial fermenters. Moreover, their association with other matrix components greatly influences cell wall properties, including the degradability of structural polysaccharides (Monties 1991). The energy value of forage thus results from their more or less high content in cell walls which have a limited and variable digestibility by microorganisms in the rumen or/and in the large intestine of cattle.

In addition to studies in woody or agronomic species, genetics and genomics of lignification have been investigated in model species such as *Arabidopsis*, zinnia, and tobacco. Only rare and scattered information is available concerning lignification in rice or in the new model grass species *Brachypodium distachyon*. Most information devoted to grass lignification is based on maize which is both one of the most economically important crops and also a classical model for plant research in genetics and genomics. In public databases, more than 1 million of EST (Expressed Sequence Tags) and an increasing amount of genomic information from almost 7000 BAC sequences are available for maize. The current maize sequencing project should achieve the objective of whole genome sequence availability by the end of the 2008, with a coverage rate of the physical map to the genetic map reaching 90% (www.maizegenome.org). Maize genomic approaches are also facilitated by well established transgenesis together with collection of tagged mutants which can be used for both reverse and forward ge-

netics screening. Moreover, because maize is easy to cross- and self-pollinate, numerous RIL progenies have been developed, with a lot of publicly available information. Complementarily, differences in lignification between grasses and dicotyledonous plants are also a tool to discover genes of specific importance in grasses. Mitchell and Shewry (2007) thus used a bioinformatics comparison between monocotyledons and dicotyledonous plants to identify candidate genes involved in the synthesis and feruloylation of arabinoxylans in grasses. Finally, understanding lignification in grasses has a theoretical and scientific interest and will contribute to the understanding of plant organization, evolution and phylogeny. Understanding lignification has also a practical interest, as the improvement of plant energy value for cattle feeding and for bio-fuel yield are both fully related to lignin quantity, monomeric composition and linkages, and to the complex relationships between lignins and cell wall carbohydrates.

BIOCHEMICAL CHARACTERISTICS OF THE LIGNIFIED GRASS CELL WALL

The lignified grass cell wall is a composite material with cellulose microfibrils, an amorphous matrix consisting of hemicelluloses (mainly glucurono-arabinoxylans) with very little pectins, and phenolics. Phenolics comprise lignins and cell wall-linked *p*-hydroxycinnamates, *p*-coumaric and ferulic (along with its array of dehydrodimers) acid derivatives. The participation of *p*-hydroxycinnamates in the cell wall composition and organization of the mature lignified tissues is certainly the most specific trait of grass lignification. Another typical characteristic is the importance of acetyl residues linked to xylan chains of glucurono-arabinoxylans. Acetyl residues may account for 5.5% of the cell wall in maize.

Lignin monomeric composition in grass cell walls

Grass lignins mostly comprise of guaiacyl (G) units, derived from coniferyl alcohol, and syringyl (S) units, derived from sinapyl alcohol, whereas *p*-hydroxyphenyl units (H), derived from *p*-coumaryl alcohol, occur as a quantitatively minor component. The H, G and S units of grass lignins are interconnected through labile β-O-4 ether bonds, and through a series of resistant carbon-carbon and biphenyl ether linkages, referred to as the condensed bonds. Native lignins of maize stalk yield thioacidolysis monomers ranging between 600 and 800 μmoles per gram of lignins. These values are three times lower than what was observed in hardwood lignins (**Table 1**), despite a similar frequency of S units in maize and hardwood lignins. Analysis of degradation products following cleavage of ether linkages by thioacidolysis has shown variable proportions of H, G, and S units between grass species (**Table 1** and Lapierre 1993). The low, but significant, amount of H units in grass lignins is a specific trait that contrasts with the trace amount recovered from constitutive dicotyledonous lignins. The impact of these minor H units on the properties of grass cell walls should not be underestimated as they increase the frequency of resistant inter-unit bonds (Cabane *et al*. 2004).

Grass lignins are also characterized by a high content in free phenolic groups (Lapierre 1993). Up to 60% of native maize lignins can be solubilized in alkali at room temperature. This massive solubilization of lignins is precluded by a mild CH_2N_2-methylation of free phenolic groups. This unusual behavior suggests that maize lignins are distributed in the cell wall as small and alkali-leachable domains. These domains have a high frequency in condensed bonds that constitute branching points (biphenyl ether bonds) and increase the abundance of terminal units with free phenolic groups. The H units essentially occur as terminal and free phenolic groups and their incorporation probably stops any further growing of the polymer. This can be explained by the high oxidation potential of H units which makes them unreactive in phenolic coupling (Brunow *et al*. 1998).

Table 1 Total yield and relative frequencies of the main monomers released by thioacidolysis of native grass lignins of mature internodes compared to herbaceous and woody dicotyledonous. H (*p*-Hydroxyphenyl), G (Guaiacyl), and S (Syringyl) are lignin units released after thioacidolysis (Monomer yields are expressed as μmoles relative to the Klason lignin content of the extractive-free sample, and H, G, and S are given as percentage of monomeric units).

	Klason lignin %	Monomer yield	H	G	S
Wheat straw	19.5	1190	4.6	43.2	52.2
Triticale straw	19.8	1610	3.0	42.0	55.0
Rye straw	18.8	1670	2.0	44.0	54.0
Maize stalk	14.8	930	2.3	37.4	60.3
Arabidopsis stem	17.3	1022	0.7	72.3	27.1
Tobacco stem	19.1	1868	0.3	51.7	48.1
Poplar wood	23.4	2310	0.3	36.7	63.0

p-Hydroxycinnamates and lignin monomeric unit relationships in grass cell walls

pCA is mainly esterified to the γ-position of the side chains of S lignin units (Ralph *et al*. 1994; Grabber *et al*. 1996) and lignified maize cell walls can contain up to 3% *p*-coumarate (Grabber *et al*. 2004). A small amount of pCA is esterified to hemicelluloses in immature tissues. However, most *p*-coumarate accretion occurs in tandem with lignification and *p*-coumarate accumulation is thus a relevant indicator of lignin deposition, particularly of S units (Grabber *et al*. 2004). Sinapyl alcohol, the precursor of S lignin units, would be enzymatically pre-acylated with pCA acid prior to their incorporation into lignin and sinapyl *p*-coumarate is thus the presumed precursor incorporated into lignin (Lu and Ralph 1999). In maize, 25 to 50% of lignin S units may be acylated by pCA. This acylation has probably a marked influence on the bonding mode of S lignin units, on the spatial organization of lignins and on their interacting capabilities with polysaccharides. While sinapyl alcohol has a pronounced tendency to be involved in β-O-4 end-wise type coupling upon peroxidasic polymerization in plant cell walls, its *p*-coumaroylation at Cγ might change its coupling modes as well as the advancement of the peroxidase-driven polymerization. As a support to this hypothesis, a model study recently established that adding sinapyl *p*-coumarate with monolignols accelerated peroxidase inactivation and this depressed lignification (Grabber and Lu 2007).

Ferulate is the major hydroxycinnamic derivative in young grass cell walls and maize cell walls can contain up to 5% ferulate monomers plus dimers (Grabber *et al*. 2004). At least 50 to 70% of alkali-labile ferulate deposition occurs during secondary wall formation and lignification (Mac Adam and Grabber 2002) and FA content plateaus as the walls come to maturity (Morrison *et al*. 1998). Ferulic units are primarily esterified to non-cellulosic polysaccharides, such as glucurono-arabinoxylans. Through their peroxidase- and/or laccase-driven oxidative coupling, ferulate and diferulates provide points of growth for the lignin polymer, mainly via ether bonds that anchor lignins to cell wall polysaccharides, and thereby direct cell wall cross-linking (Ralph *et al*. 1992; Jacquet *et al*. 1995; Ralph *et al*. 1995). Feruloylated arabinoxylans may thus be primarily responsible for the scattered distribution of lignin domains in grass cell walls. Conversely to the ester linkage of pCA to S units, FA is mainly ether-linked to the β-position of G units (Jacquet *et al*. 1995). Complementarily, the formation of ferulate dimers is all what is required to covalently couple two polysaccharide chains (Hatfield *et al*. 1999a). The presence of ferulate linked to arabinosyl substituents of arabinoxylans thus provides a convenient and reliable way of cross-linking these polysaccharide chains. Over 50% of wall ferulate can thus undergo dehydrodimerization and arabinoxylans are extensively cross-linked by ferulate dimerization in mature cell walls (Grabber *et al*. 2004).

Ester-bound pCA and FA can also undergo a photo-cata-

lyzed cyclodimerization to form ester-linked cyclobutane derivatives (Hartley and Morrison 1991), namely the truxillic acids (head to tail polymerization, dimers which are sterically favored) and the truxinic acids (head to head polymerization, dimers which are minor components). Truxillic acids involved pCA-pCA, pCA-FA, and FA-FA dimerizations and most often occur in this decreasing order. These dimers, which seem more frequent in small grain cereal straws than in maize or sorghum (Jacquet 1997), may cross-link cell wall polymers. While it is well established that cellulose and hemicellulose components are tightly associated through hydrogen bonds, the occurrence of covalent bonds between cellulose and any other cell wall constituents has not been demonstrated. Lignins can also be bound directly to non-cellulosic polysaccharides via direct ether or ester bonds resulting from opportunistic addition reactions between quinone methides and various nucleophiles.

Recently, on the basis of a thioacidolysis marker compound (Ag) and of NMR signals, Ralph *et al*. (2007) have shown that free FA could be polymerized into lignins, in addition to *p*-coumaryl, coniferyl, and sinapyl alcohols. The Ag marker thus occurred in lignins of poplar wood as nearly 1.3% of released G units (Ralph *et al*. 2007). As it was considered for lignin H units, the impact of this new minor constituent could be more important than considered on the basis of its frequency. Despite they are mostly referred to as branched 3-dimensional polymers, lignins are largely linear. This novel incorporation mode of free FA in lignins, through bis-8-*O*-4 cross-coupling, provides a new mechanism for chain branching in the polymer (Ralph *et al*. 2007). The Ag marker is also systemically released from the thioacidolysis of grass lignins, in low relative amount compared to G monomers (less than 1%). Whether this incorporation in grass lignins contributes to their high branching degree is still unknown.

DISTRIBUTION OF LIGNINS AND *P*-HYDROXYCINNAMATES IN STEM TISSUES OF GRASSES

In a maize growing internode, all tissues do not undergo lignification simultaneously, or at the same rate. Lignification appears as a spatially and temporally regulated phenomenon and the distribution of H, G, and S monomers varies among tissues and between primary and secondary cell walls (Terashima *et al*. 1986; Terashima and Fukushima 1993; Joseleau and Ruel 1997). Conducting and supporting tissues such as fibers, vessels and tracheids show an early lignification of their secondary walls, comparatively to those of parenchyma. Similarly, structure differs with respect to morphological region such as fiber, vessel and parenchyma cell walls. Lignins of the parenchyma are made of a mixture G and S lignins, with a low proportion of G units. In the tracheids of protoxylem and in the vessels of metaxylem, lignins are early found heterogeneous, with a significant proportion of H units mixed with G-S units, but a lower proportion of homopolymeric G lignins. The incorporation of H and G monomers takes place at the onset of lignification, in lignins deposited in the tricellular junctions and the medium lamella of plant cells, even if S units have been detected at the early stages of lignification (Musel *et al*. 1997). The deposition of G units with increasing proportions of S units occurs subsequently, during lignification of primary walls of parenchymatous tissues, and in primary and secondary walls of xylary tissues and sclerenchyma fibers. Xylary tissues and sclerenchyma are lignified before parenchyma and they have a higher S/G ratio and lignin content at maturity (Chesson *et al*. 1997; Joseleau and Ruel 1997). Correlatively to the accumulation of highly lignified xylary and sclerenchyma cells, which are highly enriched in S lignins, the lignin content and the S/G ratio of lignified tissues increase as the plant ages (Buxton and Russell 1988; Chen *et al*. 2002). The changes in lignin content may also be accompanied by changes in lignin structure. Although lignin and *p*-hydroxycinnamic acid deposition in individual internodes takes place over a period of weeks (Jung 2003), this process is probably completed within a few days in individual cells of internodes, but this has not yet been adequately characterized (Grabber *et al*. 2004).

Ferulate deposition has been investigated in the fourth growing internode of late maize hybrids (Jung 2003). The length of the fourth internode increased from 9 to 145 mm during the 20 days of its fast elongating period and reached finally 152 mm 45 days after the beginning of its elongation. Accumulation of ferulate followed the elongation pattern, but deposition of ester and ether ferulate derivatives continued at a high rate long after this elongation has ceased, until 80 days after the beginning of internode elongation. This duration is probably shorter in earlier lines or hybrids. Immuno-cytochemical studies with maize stems also indicate that FA is deposited in the lignified walls of secondarily thickened xylem, sclerenchyma, phloem fibers, and parenchyma tissues (Migné *et al*. 1998). In mature sorghum, total and ether-linked ferulate concentrations were greater in sclerenchyma and vascular tissues than in pith parenchyma and epidermal cells (Hatfield *et al*. 1999b). Similarly, pCA esters increased 10-fold between 20 and 80 days after the beginning of maize internode elongation, due to their presumed incorporation into lignins from sinapyl *p*-coumarate precursor and due to the late deposition of S lignin units in the cell walls (Jung 2003).

LIGNIN AND *P*-HYDROXYCINNAMATES GENES IN GRASSES

Lignin pathway enzymes are likely organized as one (or more) endoplasmic-reticulum-associated multi-enzymes complex (Winkel 2004). The precise organization of these enzymatic complexes is not elucidated, but it could be considered that the enzymes function as different metabolons, each dedicated to the production of the different phenylpropanoid compounds (Winkel 2004). These considerations are fully in agreement with the existence of multigene gene families. Each member gene in a multigene family could be differentially involved in different metabolons, with differential regulations controlled by different transcription and regulatory factors.

Genes involved in monolignol and lignin biosynthesis in grasses

The first step of lignin biosynthesis occurs after the shikimate pathway with the deamination of L-phenylalanine into cinnamic acid by a phenylalanine ammonia lyase (PAL). Maize PAL also has a tyrosine ammonia lyase (TAL) activity (Roesler *et al*. 1997) likely explaining the TAL activity observed in grasses (Higuchi *et al*. 1967) and catalyzing tyrosine deamination into *p*-coumaric acid (pCA). The hydroxylation of cinnamic acid by a cinnamate 4-hydroxylase (C4H) yields p*CA*, then converted into coumaroyl-CoA by a 4-CoA ligase (4CL). Coumaroyl-CoA is converted into caffeoyl-CoA through the formation of quinate or shikimate esters by a hydroxycinnamoyl transferase (HCT), either a hydroxycinnamoyl-CoA: quinate hydroxycinnamoyl transferase (CQT) or a hydroxycinnamoyl-CoA: shikimate hydroxycinnamoyl transferase (CST), followed by hydroxylation of these esters to caffeoyl analogues via a *p*-coumaroyl-shikimate/quinate 3'-hydroxylase (C3'H), giving chlorogenic and *trans*-(*p*-caffeoyl)-shikimic acids, respectively. Finally, formation of caffeoyl-CoA is obtained by the reverse-active HCT (Schoch *et al*. 2001; Hoffmann *et al*. 2003, 2004; Mahesh *et al*. 2007; Shadle *et al*. 2007). Whether C3'H enzymes are active on both *p*-coumaroyl-shikimate and *p*-coumaroyl-quinate or specialized on one ester is not established in maize. However, in *Arabidopsis*, the shikimate ester was converted four times faster than the quinate ester (Schoch *et al*. 2001), while in tobacco the affinity of HCT enzyme for shikimate was nearly 100-fold higher than for quinate (Hoffmann *et al*. 2003). Shikimate derivatives are therefore likely the preferential substrates towards the

biosynthesis of lignin G and S units.

Further steps of this pathway are not definitely elucidated. The lignin pathway generating G and S units was considered for a long time to be a metabolic grid, but this view has to be discarded based on the latest data. A pathway has been proposed by Chiang (2006) with the cinnamate 3-hydroxylase (C3H) acting on 4-coumarate and the 4CL acting on caffeate, but this model is not in agreement with results that established the involvement of quinate or shikimate esters of coumaroyl-CoA in plant lignin pathways and the lack of caffeate in maize. Separate pathways leading to G and S units had been proposed by Guo *et al*. (2001) and Parvathi *et al*. (2001) in the dicotyledonous alfalfa plant, with a G unit biosynthesis based on a caffeoyl-CoA O-methyltransferase (CCoAOMT) activity and an S unit biosynthesis based on a caffeic acid O-methyltransferase (COMT) activity. More recently, Chen *et al*. (2006) and Dol *et al*. (2007) have shown that down-regulation of CCoAOMT in alfalfa and *Arabidopsis*, respectively, reduced lignin content, with a significant effect on G units and an increased S/G ratio, but almost no effect on S unit yield. Contrary results were observed in CCoAOMT down-regulated tobacco with a greater reduction of S units than G units (Zhong *et al*. 1998; Chen *et al*. 2006). However, *Arabidopsis* plants down-regulated for both CCoAOMT1 and COMT1 stop their growth at the plantlet stage, with lignins mainly composed of H units (Dol *et al*. 2007). Based on these results and based on enzymatic activity (Meng and Campbell 1998; Parvathi *et al*. 2001), alfalfa and *Arabidopsi*s COMT are likely active on both 5-hydroxy-coniferaldehyde and caffeoyl-aldehyde. Conversely, CCoAOMT enzymes have a stricter affinity for CoA-ester, and more specifically for caffeoyl-CoA compared to 5-hydroxyferuloyl-CoA, and have no or very little affinity on corresponding acids (Martz *et al*. 1998; Meng and Campbell 1998; Parvathi *et al*. 2001).

Based on all available data on angiosperm lignin pathway, on data obtained from maize *bm* mutants, on maize genes found involved in the pathway, and on maize expression data, the maize lignin biosynthesis pathway appears centered on a C3'H-CCoAOMT hub with a central position of caffeoyl-CoA. One route towards G, and possibly S monolignol, is certainly based on the methylation of caffeoyl-CoA into feruloyl-CoA by a CCoAOMT. Different CCoAOMT genes are probably differentially involved in the G unit pathway with different and possibly specific involvements in each stage or tissue. Similarly, CCoAOMT genes could be differentially or specifically involved in S monomeric unit biosynthesis. Reductions of *p*-coumaroyl- and feruloyl-CoA thioesters into *p*-coumaraldehyde and coniferaldehyde are catalyzed by cinnamoyl-CoA reductases (CCR) with different members of the CCR family probably expressed at different maturity stages and/or in different tissues, as it was considered for CCoAOMT genes. Complementarily, in tobacco CCR down-regulated plants, lignin content was nearly half that of control with reduced G units, while S units were relatively retained (Piquemal *et al*. 1998; Ralph *et al*. 1998; Dauwe *et al*. 2007; Ralph *et al*. 2007). In fact, it is fairly difficult to conclude surely from observations in down-regulated plants if all members of the family are not taken into account. In maize, the five CCoAOMT genes identified to date fall into three clusters with rather low nucleic similarity between genes (Civardi *et al*. 1999; Guillaumie *et al*. 2007b) and a possibility to escape a RNA interference targeted only against a subset of members. A similar situation exists in maize for CCR genes, and more than one CCR should exist in tobacco with significantly diverging sequences.

Diverging routes to coniferyl and syringyl alcohol are a probable hypothesis. A maize CCR could catalyze the reduction of caffeoyl-CoA into caffeoyl-aldehyde, which could be subsequently converted into coniferaldehyde either by the COMT and/or by another OMT according to expression of several OMT genes observed in maize lignifying tissues (Guillaumie *et al*. 2007b). The diverging step between routes leading to the biosynthesis of G and S lignin units could be hypothesized very early in pathway. The down-regulation of the *Arabidopsis* 4CL1 gene reduced the content of G units in lignins, but did not affect the S unit content (Lee *et al*. 1997). Specific metabolic channels are possibly devoted to coniferyl and sinapyl alcohol biosynthesis, based on gene spatio-temporal regulation and involving different members of multigene families. Regarding the last steps of sinapyl alcohol biosynthesis, the hydroxylation of coniferaldehyde by a ferulate 5-hydroxylase (F5H) yields 5-hydroxy-coniferaldehyde, which is methylated into sinapaldehyde by the COMT. The unique maize COMT enzyme has, in fact, no *in vivo* activity on caffeic acid, but is active only on coniferaldehyde.

Whether separate pathways exit in maize with the biosynthesis of G units based on CCoAOMT activities and the biosynthesis of S units based on COMT/OMT activities as it was considered in alfalfa and *Arabidopsis* is not definitively answered. The more primitive appearance of CCoAOMT during plant evolution, in comparison with COMT, is in agreement with the most primitive appearance of G units comparatively to S units. Only a CCoAOMT has been found in the genome of cyanobacterium (Meng and Campbell 1998). Moreover, Li *et al*. (1999) demonstrated that a CCoAOMT gene also existed in gymnosperm (loblolly pine), with a high catalytic efficiency for caffeoyl-CoA, even if a COMT related enzyme (AEOMT), having a similar activity on acids and CoA-esters, has also been found (Li *et al*. 1997). Because several OMT of unknown substrates are expressed in maize lignifying tissues, and because the residual content of S units in *bm3* (COMT disrupted) maize mutant reaches 40% of the normal value, it could be assumed that S unit biosynthesis occurs in maize and in grasses with the involvement of both the COMT and unidentified OMT genes. Complementarily, preferred channels towards coniferyl and sinapyl monolignols, rooted early in the lignin pathway or possibly earlier in the shikimate pathway, have to be considered.

Reduction of *p*-hydroxycinnamaldehydes leads to the three *p*-coumaryl, coniferyl, and sinapyl alcohols (Boudet 2000; Boerjan *et al*. 2003; Boudet *et al*. 2003). These steps are catalyzed by cinnamyl alcohol dehydrogenases (CAD) considered as active on the three *p*-hydroxycinnamaldehydes. However, a specific sinapyl alcohol dehydrogenase (SAD) gene has been described in aspen (Li *et al*. 2001). Even though two close orthologs of the aspen gene have been found in maize (Guillaumie *et al*. 2007b), the specificity of encoded proteins towards sinapaldehyde has not yet been established in maize. The maize CAD gene Zm CAD2, which is orthologous to the EgCAD2 and OsCAD2 genes of eucalyptus and rice (Goffner *et al*. 1992; Tobias and Chow 2005), is associated with the *bm1* mutation and located in the bin 5.04 (Provan *et al*. 1997; Halpin *et al*. 1998). EgCAD2-type proteins are zinc-containing long-chain alcohol dehydrogenases active as dimers (Hawkins and Boudet 1994; Jornvall *et al*. 1987), whereas EgCAD1-type proteins are short-chain alcohol dehydrogenases (Jornvall *et al*. 1995; Goffner *et al*. 1998), which are active as monomers (Hawkins and Boudet 1994). An EgCAD1-type CAD activity has also been described in maize by Kanazawa *et al*. (1999) and a corresponding gene ZmCAD1 was recently mapped in bin 5.04 close to the *bm1* gene (Génoplante unpublished data). The function of EgCAD1-type enzymes in the constitutive lignin pathway is not completely explained, but one such gene was recently proven to be also involved in the synthesis of coniferyl alcohol (Damiani *et al*. 2005). Complementarily, AtCAD1 was observed expressed in the early lignification stages of *Arabidopsis* and allowed a partial restoration of lignification in CAD C and CAD D down-regulated plants (Eudes *et al*. 2006). The hydroxylation and methylation steps giving sinapyl alcohol are also considered to occur at the alcohol level, as F5H and COMT are likely active on coniferyl and 5-hydroxy-coniferyl alcohols, respectively.

The three resulting *p*-hydroxycinnamyl alcohols are transported as still unidentified conjugates from the cytosol

to the apoplast where they undergo dehydrogenative polymerization mainly via peroxidases and/or laccases to form lignins (Boudet 2000; Christensen *et al*. 2000; Boerjan *et al*. 2003). The mode of condensation of monolignols is controversial, as it is mostly often considered occurring after free radical coupling, but it was also considered to occur through an ordered radical coupling driven by dirigent proteins (Davin and Lewis 2000). Whereas peroxidases have been considered during years as the unique class of oxidases involved in lignin polymerization, new results, out of which the sequencing of EST and expression studies based on lignifying tissues, have shown that other oxidases and particularly laccases could be involved in lignification. Peroxidases and laccases belong to large multigene families and it has been to date difficult to assign a specific function to any particular oxidase (Boudet 2000). Moreover, sinapyl alcohol is far more rapidly oxidized in presence of *p*-coumarate, which is oxidized by peroxidases and transfers the radical to sinapyl alcohol (Boudet 2000). Two maize peroxidases (ZmPox2 and ZmPox3) have been shown to localize in vascular and lignifying tissues (de Obeso *et al*. 2003). Complementarily, ZmPox2, ZmPox3, and three new maize peroxidase genes, orthologous to zinnia peroxidase genes, were shown significantly expressed in maize lignifying internodes of silking plants (Guillaumie *et al*. 2007b). In the same experiment, three laccase genes, orthologous to poplar and zinnia laccase genes, were simultaneously expressed, one of which with a 2-fold higher level than the most expressed peroxidase gene.

A less direct pathway for monolignol to enter the lignin polymer has possibly also to be considered, based on the occurrence of coniferin (coniferyl alcohol 4-*O*-β-D-glucoside) which is likely a stock compound. In the primitive tree *Ginkgo biloba*, coniferin was shown to be oxidized into coniferaldehyde glucoside by a NADP-dependent alcohol dehydrogenase, deglucosylated by a β-glucosidase into coniferaldehyde, and finally converted into coniferyl alcohol and incorporated into lignin (Tsuji *et al*. 2005). In magnolia (plant with a coniferin pool), oleander or eucalyptus (plants without a coniferin pool), most of coniferin aglycone used for lignin biosynthesis via an aldehyde form was incorporated mostly in syringyl, but not in guaiacyl lignins (Tsuji *et al*. 2004; Tsuji and Fukushima 2004).

Genes involved in *p*-coumarate and ferulate biosynthesis in grasses

The biosynthesis of p*CA* is not questioned, even if a specific set of members of multigene families could be involved in its biosynthesis. However, while sinapyl alcohol is presumably pre-acylated with pCA acid prior to its incorporation into lignin (Lu and Ralph 1999; Grabber and Lu 2007), the involved acyltransferases are yet unknown. Conversely, the pathway allowing ferulic acid (FA) biosynthesis is still largely unknown.

In *Arabidopsis*, Nair *et al*. (2004) established that the ref1 mutant, which has a reduced content in soluble sinapate esters, was affected in a sinapaldehyde dehydrogenase (ALDH2C4, At3g24503), and that the REF1 protein exhibited both sinapaldehyde and coniferaldehyde dehydrogenase activities. Sinapic and probably ferulic acids in *Arabidopsis* thus (partially) derived from oxidation of the corresponding aldehydes, rather than acting as precursors of those aldehydes. Whether this ALDH pathway also exists in grasses, or whether it is limited to *Brassiceae* or a subset of dicotyledons plants, is currently not established. In maize, at least eight ALDH genes have been described (Skibbe *et al*. 2002), including the ZmRF2A gene involved in fertility restoration of the Texas cytoplasmic male sterility and the cytosolic ZmRF2C, ZmRF2D1 and ZmRF2D2 genes (Skibbe *et al*. 2002; Nair *et al*. 2004). In maize young or older tissues (Guillaumie *et al*. 2007b), gene expression level for investigated ALDH was rather low, except a slightly higher expression for the ALDH *Arabidopsis* REF1 ortholog AY 109842, first found in anther and pollen libraries and originally annotated as BADH (betaine aldehyde dehydrogenase). This gene corresponds to the maize locus umc1760, previously mapped in bin 7.05. Its closest orthologous gene in rice is also annotated as a BADH (BAC21357). In *Arabidopsis*, the closest orthologous gene (At3g66658) encoded protein that is now considered as a putative ALDH (ALDH22A1), belonging to a novel plant ALDH family (Kirch *et al*. 2004, 2005). In maize, the putative involvement of ALDH enzymes in ferulate biosynthesis has still to be considered.

In alfalfa, the content in FA, which is present in the cell wall with an amount nearly 100 times lower than in maize, is significantly decreased in C3'H down-regulated plants, but not in CCoAOMT down-regulated plants (Chen *et al*. 2006). Even if these data did not establish if all members of the CCoAOMT family were down-regulated or not, they probably established that ferulate originates either from a specific and diverging CCoAOMT escaping the RNA interference or more probably from a route branching before the CCoAOMT step. Complementarily, in addition to variation in lignin content and decrease of lignin G units in tobacco CCR down-regulated plants, an increase in ferulate content of the cell wall (up to 10-fold) was observed (Piquemal *et al*. 1998; Ralph *et al*. 1998; Dauwe *et al*. 2007; Ralph *et al*. 2007). FA biosynthesis pathway appeared closer to the one of syringyl alcohol than to the one of coniferyl alcohol.

In any way, the pathway leading to FA still remains largely questionable in maize and grasses. Results obtained with the maize *bm3* mutant strongly suggest that FA is not biosynthesized by a COMT-catalyzed methylation of a caffeic precursor. The disruption of the COMT gene neither affects FA content nor probably its biosynthesis (Barrière *et al*. 2004c), even if the lower lignin content in the *bm3* mutant may increase the yield of alkali-releasable FA (Grabber *et al*. 2000b). From investigations in maize or wheat stem tissues allowing the recovery of *p*-coumarate and ferulate, no caffeate was found (Fry *et al*. 2000; Benoit *et al*. 2006; INRA Lusignan unpublished data). Complementarily, in a mapping progeny derived from a cross between *Coffea pseudozanguebariae* and *C. liberica* (Campa *et al*. 2003), a QTL closely linked to a CCoAOMT gene was shown to affect the content in caffeoyl-quinic (chlorogenic) acid, but has no effect on the content in feruloyl-quinic acid. A lower CCoAOMT activity or efficiency was then considered to increase the pool of chlorogenic acid, but no satisfactory hypothesis explained the absence of effect on feruloyl-quinic acid content. Obel *et al*. (2003) showed that feruloyl-glucose can be a precursor for the intracellular feruloylation of arabinoxylans when radio-labelled FA was given to suspension-cultured wheat cells. However, the formation of feruloyl-glucose might also occur as storage or detoxification processes when cells are fed FA, in the same way as monolignol glucosides are considered to be storage and/or the transport forms of monolignols (Lim *et al*. 2005a, 2005b; Escamilla-Trevino *et al*. 2006). According to Fry *et al*. (2000), the *p*-coumarate to caffeate and ferulate conversion could involve conjugates of the acids, including possibly CoA-esters. Correlatively, a putative feruloyl-CoA-arabinoxylan-trisaccharide *O*-hydroxycinnamoyl transferase activity (Yoshida-Shimokawa *et al*. 2001) has been found *in vitro* in suspension-cultured rice cells fed feruloyl-CoA and arabinoxylan-trisaccharide (AXX) towards the formation of feruloyl arabinoxylan-trisaccharide (FAXX). However, if data of Obel *et al*. (2003) suggested that feruloyl-CoA was in fact the precursor of feruloylated proteins, it could also be hypothesized that feruloyl-CoA would be only an intermediary feruloyl donor towards the formation of FAXX. The results of Fry *et al*. (2000) showing incorporation of [^{14}C]cinnamates into arabinoxylan-bound ferulate and diferulates in maize cell cultures did not similarly clearly identified feruloyl-CoA as an intermediate between cinnamate and cross-coupled ferulate. Taking into account the results of Chen *et al*. (2006) on alfalfa showing a ferulate pathway branching after C3'H and before CCoAOMT steps, and because coumaroyl-CoA was now proven to be converted into

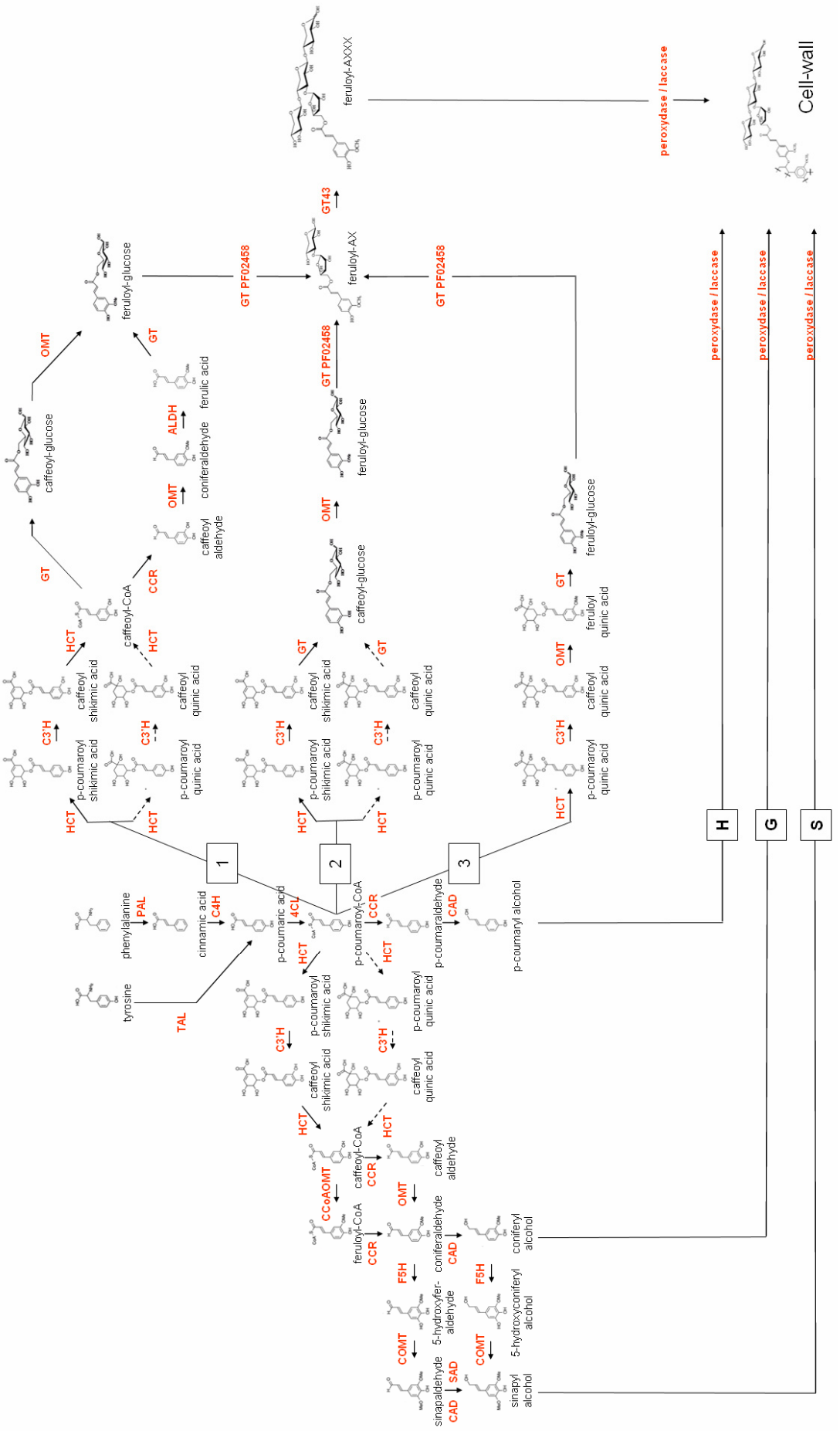

Fig. 1 Putative pathways for lignins and ferulic acid biosynthesis in maize and grasses. ALDH: aldehyde dehydrogenase; AXXX: arabinoxylan; COMT: caffeic acid O-methyltransferase; CCoAOMT: caffeoyl-CoA O-methyltransferase; CCR: cinnamoyl-CoA reductase; CAD: cinnamyl alcohol dehydrogenase; C3H: coumarate 3-hydroxylase; C4H: cinnamate 4-hydroxylase; 4CL: 4-coumarate:coenzyme A ligase; F5H: ferulate 5-hydroxylase; G: lignin guaiacyl unit; GT: glucosyl transferase; H: *p*-hydroxyphenyl lignin unit; HCT: hydroxycinnamoyl-CoA transferase; OMT: O-methyl transferase; PAL: phenylalanine ammonia lyase; S: syringyl lignin unit; SAD: sinapyl alcohol dehydrogenase.

caffeoyl-CoA through the formation of quinate or shikimate esters, it could be considered that either caffeoyl-quinic acid, caffeoyl-shikimic acid, or caffeoyl-CoA is the starting point of a specific pathway towards the biosynthesis of FA in grasses. While caffeoyl- and feruloyl-quinic acids, includeing 5-*O*-caffeoyl- and 5-*O*-feruloyl-quinic acids, have been found in several plants (Ding *et al*. 2001; Campa *et al*. 2003; Niggeweg *et al*. 2004; Clifford *et al*. 2006, 2007), the corresponding feruloyl-shikimic derivatives were not described. A methylation step towards feruloyl derivatives could involve caffeoyl-quinic acid and a still unknown OMT.

Three routes leading to maize and grass ferulate derivatives can thus be hypothesized (**Fig. 1**). Feruloylated arabinoxylan (AX) might originate from caffeoyl-CoA. A glucosyl transferase could convert caffeoyl-CoA into caffeoyl-glucose, converted into feruloyl-glucose by an unknown OMT. As it was considered for S unit biosynthesis, caffeoyl-CoA might also be converted into caffeoyl-aldehyde in a CCR-catalyzed reaction followed by a methylation step with an unknown OMT gene, different from COMT because FA content is not altered in *bm3* maize. Coniferaldehyde would be converted into FA in an ALDH-catalyzed reaction, and converted subsequently into feruloyl-glucose by a glucosyl transferase. Another route might be hypothesized without the involvement of caffeoylCoA. Caffeoyl-glucose might originate directly from caffeoyl-shikimic or caffeoyl-quinic acids, after a reaction catalyzed by a glucosyl transferase, with the methylation step on caffeoyl-glucose. Finally, the route toward ferulate derivatives might originate from caffeoyl-quinic acid. Feruloyl-quinic acid might occur from the methylation of caffeoyl-quinic acid. In this case feruloyl-glucose would derive from feruloyl-quinic acid with a glucosyltransferase-mediated reaction. In these three putative pathways, FA biosynthesis will effectively be free of any CCoAOMT activity.

For AX feruloylation steps, Mitchell and Shewry (2007) identified candidate genes specifically expressed in grasses, based on a bioinformatics approach on rice, wheat and barley EST, comparatively to dicotyledons. One of the most differentially expressed groups gathered grass genes of the Pfam family PF02458 which are the strongest candidates for encoding enzymes with an AX feruloyl transferase function. Maize and grass feruloylation of arabinoxylans would be catalyzed by one or several genes of this PF0258 family, mediating the transfer of a feruloyl group from feruloyl-quinic acid or feruloyl-glucose onto an AX chain. Simultaneously with genes of the PF0258 family, genes of the glycosyl transferase GT43 and GT47 families were shown as specifically expressed in grass with β-1,4-xylan synthase and xylan α-1,2- or α-1,3-arabinosyl transferase functions, respectively, allowing the lengthening of xylans chains and the formation of AX(X) groups. Complementarily, specific grass feruloyl-AX β-1,2-xylosyl transferases of the GT61 family could allow the branching of xylan chain. Genes of the GT47 family could allow the transfer of an arabinosyl residue onto an X(X) chain. Finally, feruloyl glycosides are exported to the maturing wall and the cross-linking of feruloyl arabinoxylan further involves an active oxidative coupling mechanism via peroxidases/H_2O_2 and/or laccases/O_2.

The rice mutant Fukei71 over-accumulates polysaccharide-linked ferulate in parenchyma cell wall in which lignification is normally missing (Nishikubo *et al*. 2000). The Fukei71 mutation occurs in the still unidentified D50 gene, which has been mapped on rice chromosome 2 in a 2.2 cM area close to the centromer (Sato *et al*. 2002). Very few candidate genes are available in this area according to the TIGR database (www.tigr.org) out of which the transcription factor Os02g26430 (OsWRKY42) with the conserved WRKY and zinc finger domains. WRKY transcription factors encode proteins mostly involved in disease-resistance responses, but also controlling diverse developmental and physiological processes (Eulgem *et al*. 2000; Qu and Zhu 2006; Qiu *et al*. 2007). The OsWRKY42 gene is thus a putative candidate for the Fukei71 mutation, but also a putative candidate involved in regulation of feruloylation in grasses. Whatever could be the D50 gene, the Fukei71 mutation is a clue more illustrating the possible independence and specificity of pathways towards lignin biosynthesis and ferulate biosynthesis.

In any way, the ferulic moiety of the feruloylated arabinoxylans in maize and grasses originates from the hydroxycinnamoyl-CoA channel and candidates for the transfer of feruloyl groups onto arabinoxylans and arabinoxylan chains are now available. It remains to be found out whether the branching point is effectively caffeoyl-CoA or caffeoyl quinate (or shikimate). Moreover, OMT gene(s) involved in the methylation step are still unidentified and whether coniferaldehyde and ALDH significantly contribute to feruloyl compound biosynthesis in grasses is still unknown. A pathway branched after the C3'H step and before the CCoAOMT step, with a more or less important involvement of ALDH genes, is nevertheless a plausible hypothesis. The discovery of the methylation step will answer all these questions.

Multigene family in lignin biosynthesis in grasses

Most genes of the lignin pathway belong to multigene families, with probable expression of the different members in different tissues, at different stages, and/or driving the biosynthesis of different monomers. Corroborating this view, the independent pathway in *Arabidopsis* and alfalfa towards coniferyl and sinapyl alcohol biosynthesis (Lee *et al*. 1997; Guo *et al*. 2001; Parvathi *et al*. 2001; Dol *et al*. 2007) could correspond to independent metabolic channels driven by different regulating factors and involving different members of multigene families. Based on data of Guillaumie *et al*. (2007b), at least 37 genes could be involved in monolignol biosynthesis in maize, while only 10 steps were considered from phenylalanine to monolignols (**Table 2**). This non-exhaustive list of 37 maize genes has been established from expression of their zinnia orthologs during *in vivo* xylogenesis (Pesquet *et al*. 2005a), from EST expressed in lignifying tissue of maize, and from orthologs of EST expressed in lignifying tissue of *Arabidopsis* or pine. In each family, gene number is variable and ranges from one to seven, but all the members are likely not yet known in all families. Only one C3'H gene was found to date in maize, with a moderate level of expression. Since the demonstration of the coupled involvement of HCT and C3'H gene in this hydroxylation step has only recently been established in *Arabidopsis* and tobacco (Schoch *et al*. 2001; Hoffmann 2003), it might still be considered that the complete orthologous pathway is not fully known in maize and that more than the only currently known C3'H gene could exist in maize or other grasses. However, in *Arabidopsis* the CYP98A3 gene, encoding the cytochrome P450 enzyme C'3H, is present as a single copy (Schoch *et al*. 2001). Abdulrazzak *et al*. (2006) have confirmed, based on a study of a disrupted *Arabidopsis* C3'H mutant, that this meta-hydroxylation step was non-redundant. Moreover, none of the two other *Arabidopsis* CYP98 genes (CYP98A8 and CYP98A9) metabolized the quinate or shikimate ester of p*CA* (Schoch *et al*. 2001). Based on *in silico* analyses in Gramene and TIGR databases (www.gramene.org and www.tigr.org), two putative cytochrome P450 98A1 rice genes (Os10g12080 and Os5g41440) are found as orthologous to both *Arabidopsis* and maize C'3H genes. Similarly, only one maize F5H gene (CYP84A1, Meyer *et al*. 1996) was investigated in Guillaumie *et al*. (2007b). However, at least a second gene exists in the maize genome (Génoplante unpublished data) in agreement with the presence of three CYP84A1 genes as orthologs of this maize F5H in the rice genome (Os10g36848, Os03g02180, and Os06g24180). Conversely, all available data established that only one COMT gene exists in the maize genome, as it has been corroborated by several observations including those on the *bm3* mutant (Grand *et al*. 1985; Vignols *et al*. 1995; Barrière *et al*. 2004c). In rice, only one COMT gene (Os08g06100) was observed as orthologous to the maize COMT gene.

Table 2 Lignin pathway genes normalized expression values in ear and basal internodes of silking plants of the maize INRA line F2. Genes are ranked within each family according to their expression in the ear internode. Expressions equal or lower than 3000 correspond to the zero value. Results are based on data of Guillaumie *et al*. (2007b).

Genes of the lignin pathway	mRNA	Internodes	
		Ear	Basal
Phenylalanine / Tyrosine ammonia lyase	AC185453	207907	32836
Phenylalanine / Tyrosine ammonia lyase (MZEPAL)	AY103947	187353	38154
Phenylalanine / Tyrosine ammonia lyase	AY106831	102659	31100
Phenylalanine / Tyrosine ammonia lyase	AY104679	10421	5944
Cinnamate 4-hydroxylase (C4H1)	AY104175	66372	28254
Cinnamate 4-hydroxylase (C4H2)	CF647652	19988	30822
4-coumarate coenzyme A ligase (4CL1)	AY105108	22522	6154
4-coumarate coenzyme A ligase (4CL2)	AX204867	10707	11347
4-coumarate coenzyme A ligase (4CL3)	DT948265	10286	10709
4-coumarate coenzyme A ligase (4CL5)	AY106966	10324	5159
4-coumarate coenzyme A ligase (4CL4)	BT017473	8180	5424
Hydroxycinnamoyl-CoA transferase (HCT1)	AY109546	11068	5908
Hydroxycinnamoyl-CoA transferase (HCT2)	DR807341	10690	9178
p-Coumaroyl-shikimate/quinate 3'-hydroxylase (C3'H)	AY107051	10718	7621
Caffeoyl CoA O-methyltransferase (CCoAOMT5)	AY108449	51550	13430
Caffeoyl CoA O-methyltransferase (CCoAOMT2)	AJ242981	47434	19746
Caffeoyl CoA O-methyltransferase (CCoAOMT3)	AY104670	23740	32846
Caffeoyl CoA O-methyltransferase (CCoAOMT1)	AJ242980	21976	7869
Caffeoyl CoA O-methyltransferase (CCoAOMT4)	AI855419	13947	14245
Cinnamoyl CoA reductase (CCR1, ZmCINNRED)	X98083	37894	11443
Cinnamoyl CoA reductase (CCR)	AY108351	13755	21430
Cinnamoyl CoA reductase (CCR)	AY103770	11730	6880
Cinnamoyl CoA reductase (CCR)	AI881365	9973	7397
Cinnamoyl CoA reductase (CCR)	DV490994	8886	5095
Cinnamoyl CoA reductase (CCR)	BT018028	8736	5281
Cinnamoyl CoA reductase (CCR)	AI737052	8414	4766
Cinnamoyl CoA reductase (CCR2)	Y15069	8776	7135
Ferulate 5-hydroxylase (F5H1)	DR966008	45662	13754
Caffeic acid O-methyltransferase (COMT)	M73235	142203	39113
Cinnamyl alcohol dehydrogenase (CAD, EgCAD2 type)	Y13733	30285	9569
Cinnamyl alcohol dehydrogenase (putative CAD)	AY107977	13998	6463
Cinnamyl alcohol dehydrogenase (putative CAD)	AY110917	9826	6158
Cinnamyl alcohol dehydrogenase (putative CAD)	CX129557	8260	5537
Cinnamyl alcohol dehydrogenase (CAD, EgCAD1 type)	AY106077	16082	16995
Sinapyl alcohol dehydrogenase (SAD)	AY104431	17398	6445
Sinapyl alcohol dehydrogenase (SAD)	CD995201	9165	4230
Peroxidase (ZmPox)	AY106450	19551	7099
Peroxidase (ZmPox2)	AJ401275	10903	20634
Peroxidase (ZmPox)	AY110228	10476	15081
Peroxidase (ZmPox)	BG838000	9637	5657
Peroxidase (ZmPox3)	AJ401276	9180	4716
Laccase	BG842157	46998	83977
Laccase	AI491689	13703	21387
Laccase	no	9362	4218
Means of gene expression	-	31334	14937

GENES RELATED TO THE PHENYLPROPANOID PATHWAY IN GRASSES

Genes upstream the lignin pathway

Lignin biosynthesis is also dependent on the availability of substrates upstream the phenylpropanoid pathway. The shikimate pathway links the carbohydrate metabolism to the biosynthesis of aromatic amino acids (Phe, Tyr, and Trp) and, consequently, to the phenylpropanoid and lignin pathways. The first step of the shikimate pathway is catalyzed by the 3-deoxy-d-arabino-heptulosonate 7-phosphate synthase (DAHPS). In the rice mutant Fukei71, one DAHPS is over-expressed in close correlation with one OsPAL over-expression, suggesting a coordinate regulation in the phenylpropanoid and shikimic pathways (Mase *et al*. 2005; Sato *et al*. 2006). Similarly, a chorismate mutase, which is one of the last enzymes of the shikimate pathway, was under-expressed in *bm1* and *bm2* maize young stems (Guillaumie *et al*. 2007a). The carbon flow in the shikimate pathway, the regulations of the shikimate pathway genes, and the phenylalanine supply to PAL enzymes may thus directly affect the phenylpropanoid biosynthesis.

The S-adenosyl-L-methionine cycles

Similarly as the shikimate pathway for phenylalanine, the methionine pathway is involved in the biosynthesis of substrates of lignins and hydroxycinnamates with S-adenosyl-L-methionine (SAM or AdoMet) since *O*-methyltransferases are SAM-dependent. The methylation of lignin precursors consumes large amount of methyl groups (van der Mijnsbrugge *et al*. 2000) and the formation of SAM from methionine and ATP is catalyzed by an S-adenosyl-methionine synthetase (SAMS). These transmethylation reactions based on SAM release S-adenosyl-homocysteine (SAH or Ado Hcy), which is a strong competitive inhibititor of COMT and CCoAOMT (Ravanel *et al*. 1998), and that has to be promptly recycled for proper methylation of lignin precursors. SAH is thus recycled into homocysteine and adenosine by an S-adenosyl-homocysteine hydrolase (SAHH) while an adenosine kinase (ADK) mediates the recycling of adenosine into adenosine monophosphate (Ranocha *et al*. 2000,

2001; Moffatt *et al*. 2002), catalyzed by a homocysteine methyltransferase (HMT) with the conversion of 5-methyl-tetrahydrofolate into tetrahydrofolate (THF). This cycle is dependent of another related methyl cycle in which THF converted into 5,10-methylene-THF with a simultaneous conversion of serine into glycine in a reaction catalyzed by a glycine hydroxymethyltransferase. The 5,10-methylene-THF is reduced into 5-methyl-THF by a methylenetetrahydrofolate reductase (MTHFR).

Additionally, a "futile" cycle has been shown in plants with the synthesis of S-methylmethionine (SMM) by a methyl transfer from SAM to methionine and the release of SAH (Ranocha *et al*. 2001). SMM is reconverted to methionine by transferring a methyl group to homocysteine. These irreversible reactions are catalyzed by S-adenosylmethionine:methionine S-methyltransferase (MMT) and homocysteine S-methyltransferase (HMT), respectively. Four HMT (ZmHMT1-4) have been identified in maize (Ranocha *et al*. 2001), but their substrate specificities have not been yet established. As the two negative feedback effects inhibiting MTHFR or SAMS activities are missing in plants, the SMM cycle is considered to be the main mechanism regulating the SAM pool in the absence of feedback loops between SAM and the enzymes involved in its synthesis in plants, conversely to what occurs in other eukaryotes (Ranocha *et al*. 2001; Kocsis *et al*. 2003). The SAM/SAH ratio is crucial to methylation reactions as SAH is a potent competitive inhibitor of methyltransferase enzymes (Kocsis *et al*. 2003) and is consequently of probable high significant influence on plant lignification.

In lignifying tissues, and based on a proteomic approach, PAL, CCoAOMT, and COMT profiles were highly correlated with SAMS, HMT, and MTHFR profiles (Vincent *et al*. 2003). Similarly, two SAMS (BT018468 and BG837557) were simultaneously expressed with COMT and CCoAOMT in young and silking plants of maize (Guillaumie *et al*. 2007b). Kirst *et al*. (2004) also observed a highly significant association among the expression levels of phenylpropanoid genes including F5H, CCoAOMT, OMT, shikimate genes (DAHP and chorismate mutase), and methionine related genes (SAMS, HMT, and SAH) in a eucalyptus progeny. Moreover, from yeast engineering data, an allosteric inhibition of MTHFR by SAM could control the methyl neogenesis flux (Roje *et al*. 2002) and consequently the availability of methionine and SAM for the lignin pathway. Complementarily, ADK and SAHH had jointly a higher expression in growing organs in which the highest SAM utilization is devoted to cell wall formation (Pereira *et al*. 2007). These sets of inter-dependent cycles may therefore impact significantly the efficiency of methylation reaction in the lignin pathway and correlatively the quantity of lignins, the S/G ratio, and the ferulate contents.

Genes putatively involved in the regulation of lignin pathway genes

Several genes involved in the regulation of lignin biosynthesis have been described in different species, but to date, very little is known in maize and grasses. The regulation of phenylpropanoid biosynthesis was the first role identified for a plant R2R3-MYB transcription factor (Paz-Ares *et al*. 1987). Eucalyptus EgMYB2 thus encoded a protein which was shown to be a transcriptional activator binding specifically *cis*-regulatory regions of EgCCR and EgCAD2 genes (Goicoechea *et al*. 2005). Several MYB genes has been described in maize (Franken *et al*. 1994; Guillaumie *et al*. 2007b) but only ZmMYB31 and ZmMYB42 was proven to be related to the lignin pathway as their over-expression induced a down-regulation of the maize COMT gene (Fornale *et al*. 2006). The repressing effect on COMT was stronger with ZmMYB31 than with ZmMYB42, but ZmMYB42 also negatively regulated the expression of C4H and CAD genes. Similarly, after the description of the involvement of a LIM transcription factor (Ntlim1) in tobacco lignification by Kawaoka and Ebinuma (2001), five LIM factors were shown expressed in maize lignifying tissue (Guillaumie *et al*. 2007b).

Transcription factors are also involved in lignified tissue patterning. Maize orthologs to the MYB Altered Phloem Development (APL) gene of *Arabidopsis*, which promotes phloem differentiation and represses xylem differentiation during vascular development (Bonke *et al*. 2003), were shown to be expressed in maize tissue (Guillaumie *et al*. 2007b). Orthologs of rice bZIP factors RF2a and RF2b genes, which act as activators of phloem-specific gene expression (Yin *et al*. 1997), were also found expressed in maize lignifying tissue. Several Class III homeodomain leucine-zipper (HD-Zip III) genes were proven to be expressed in maize lignifying tissues (Guillaumie *et al*. 2007b). HD-Zip III proteins are transcription factors under the control of miRNA regulation (Emery *et al*. 2003), which are involved in the asymmetric patterning of xylem and phloem in vascular bundles, promoting vascular cell division, and differentiation of interfascicular fibers and secondary xylem (Ratcliffe *et al*. 2000; Baima *et al*. 2001; Carlsbecker and Helariutta 2005). Correlatively, the maize ZmmiR166 miRNA accumulates in phloem and regulates the maize rolled leaf1 gene (rld1), which encodes a HD-ZIP III transcription factor (Juarez *et al*. 2004). Complementarily, the use of artificial zinc finger chimeras, containing either an activation or a repression domain towards the *Arabidopsis* At4CL1 promoter region, resulted in a nearly 30% increase of lignin content with an ectopic lignin distribution, or a nearly 40% decrease of lignin content with a decrease of the S/G ratio, respectively (Sanchez *et al*. 2006). Finally, two continuous vascular ring (COV1) orthologous genes were expressed in maize lignifying tissues (Guillaumie *et al*. 2007b) when the *Arabidopsis* COV1 mutant exhibited a great increase in stem vascular tissue instead of the inter-fascicular regions (Parker *et al*. 2003).

Genes putatively involved in the transport and storage of monolignols and hydroxycinnamates

Monolignol glucosides are considered to be storage and/or transport forms of monolignols. Three uridine-diphosphate-glucosyltransferases (UGT) have been identified in *Arabidopsis* (Lim *et al*. 2005a), capable of glusosylating coniferyl and sinapyl alcohols (UGT72E2, 72E3) or specifically aldehydes (UGT72E1). However, none of these UGT was expressed in stems, but only in roots and/or leaves (Lanot *et al*. 2006). The release of monolignol aglycone from its glucosidic form at the cell wall for subsequent lignin polymerization is thought to be mediated by specific glucosidases. A coniferin β-glucosidase was first identified from a pine xylem library by Dharmawardhana *et al*. (1995). Recently, *Arabidopsis* BGLU45 and BGLU46 β-glucosidases, strongly expressed in lignifying organs, were shown to encode proteins with narrow specificity towards the three monolignol glucosides (Escamilla-Trevino *et al*. 2006). Several β-glucosidase genes are expressed in maize lignifying internodes (Guillaumie *et al*. 2007b), indicating probably their involvement in the hydrolysis of monolignol glucosides into their reactive forms and thus that monolignol storage and transportation occurr in maize and grasses as glycosylated forms. These β-glucosidases share sequence homology with the β-glucosidase ZmGlu1, involved in defense against the European corn borer and catalyzing the hydrolysis of DIMBOAGlc (2-O-β-D-glucopyranosyl-4-hydroxy-7-methoxy-1,4-benzoxazin-3-one) into DIMBOA and glucose (Czjzek *et al*. 2000).

GENETIC VARIATION OF PHENYLPROPANOID CONTENTS IN GRASS CELL WALLS

Genetic variation for phenolic traits as a consequence of brown-midrib mutations

Genetic variation for lignin content and structure was first established in the maize brown-midrib (*bm*) mutants in the

sixties (Kuc and Nelson 1964; Gee *et al*. 1968; Kuc *et al*. 1968), with detailed characterization since the end of the eighties (Lapierre *et al*. 1988; Chabbert *et al*. 1994; Vermerris *et al*. 2002; Barrière *et al*. 2004c). Among the four *bm* mutants, *bm1* and *bm3* mutants have been more thoroughly investigated, as they have the most important effects on lignin content and cell wall degradability, and because genes related to these mutations have been discovered or suspected, while they are still unknown for *bm2* and *bm4*. The most distinguishing trait in *bm1* mutant plants, which are altered in a CAD2 gene expression (Halpin *et al*. 1998), is the significant incorporation of cinnamaldehydes (mainly coniferaldehyde) in constitutive lignins. Moreover, these coniferaldehyde units incorporate into *bm1* lignins not only through labile ether bonds, but also through resistant biphenyl structures (Barrière *et al*. 2004c). Even if no "brown-midrib" mutant has been described in rice, the GOLD HULL AND INTERNODE2 (GH2) mutation in rice induces a reddish-brown pigment in panicle (or hull), internode, and basal leaf sheath at heading stage. The GH2 mutant was recently shown to be a mutant of the OsCAD2 (Os02g09490) gene which is the closest ortholog of the ZmCAD2/*bm1* maize gene (Zhang *et al*. 2006). The most significantly modified trait related to cell wall phenolics in *bm2* is the substantial reduction of β-O-4-linked G units, whereas no changes were observed for β-O-4-linked S units. Complementarily, a significant decrease in etherified FA released after alkaline hydrolysis was also observed which could be correlated to the lower availability of G units, with only a slight shift in esterified FA (Barrière *et al*. 2004c). The *bm3* mutant has been most intensively studied, probably due to its very low lignin content which is reduced by 25 to 40% (and its high efficiency in cattle feeding). Kuc and Nelson first established in 1964 that the frequency of S units was heavily reduced, and suspected the occurrence of additional not yet detected units. These additional units were identified latter as 5-hydroxyguaiacyl (5-OH-G) units (Lapierre *et al*. 1988), which were also shown to be involved in novel benzodioxane structures in lignins (Marita *et al*. 2003). The *bm3* mutation occurs in the second exon of the unique maize COMT gene (Vignols *et al*. 1995), disrupting the S unit pathway. The maize *bm4* mutant is to date only characterized by its brown-midrib leaves and its reduced lignin content, without any abnormal trait highlighted in the lignin polymer.

Genetic variation for phenolic traits in normal maize

Large variation for phenolic traits were shown between normal inbred lines or hybrids (Dhillon *et al*. 1990; Grotewold and Peterson 1994; Lundvall *et al*. 1994; Méchin *et al*. 2000; Fontaine *et al*. 2003a, 2003b; Frey *et al*. 2004). Lignin content and structure, esterified pCA content, esterified and etherified FA contents are thus subject to significant genetic variation in grass cell walls (Casler and Jung 1999; Fontaine *et al*. 2003b). Among maize lines, a variation close to 50% and 30% of average values is observed for ADL/NDF and KL/NDF lignin contents, respectively (**Table 3**), in agreement with results of Lundvall *et al*. (1994) obtained with later lines (B73 type). In correlation with lignin content variation, a 2-fold variation was observed for pCA content. Nearly similar ranges of variation were observed for both esterified and etherified FA. Variation for the recovery yield of each H, G or S lignin-derived monomer after thioacidolysis or nitrobenzene oxidation was similarly important between maize lines, inducing consequently variation in the corresponding S/G ratio.

However, observed differences between genotypes for phenolic traits may relate to fundamentally different mechanisms. Differences in phenolic traits may be explained by genetic mechanisms directly involved in lignification, with more or less efficient enzymes in the lignin pathway or its upstream components, with more or less strong, even silent, regulatory genes, with variable duration of gene expression. However, within a stem, variable proportions in the different tissues, which have specific ratios in H, G, and S lignin units and variable intensities of lignification, induce consequently differences in phenolic traits between lines.

Genetic variation for tissue histology in maize

Genetic variation in lignification based on histological parameters has been little studied in grass. Two studies have reported on the occurrence of thinner cell walls in *bm3* maize mutants (Grenet and Barry 1991; Goto *et al.* 1994). Wilson and Hatfield (1997), investigating simultaneously structural and chemical changes of cell wall types during stem development, considered that poor digestion of secondary walls is *in vivo* due to limits imposed by anatomical structure. Wilson and Mertens (1995) considered that, in grasses, the anatomical structures of cells and tissues might be more important than wall chemistry in determining rate and extent of fiber digestion *in vivo*, because anatomical structure significantly influences wall accessibility to rumen microorganisms. It is fairly difficult to conclude whether anatomical or biochemical traits are the most important for cell wall digestibility, all the more that lignin content and structure is different in each type of lignified tissue.

Genetic variation for histological factors has been investigated by Méchin *et al*. (2000, 2005) based on Fasga- and Mäule-stained maize stem sections in a set of 22 inbred lines. The cortical area, the thickness of cell layers in the cortical sclerenchyma, the number of cell layers in the cortical sclerenchyma, the non-lignified and lignified areas (Fasga-stained blue and red areas, respectively), and the lignified areas discriminated on the basis of their G and S content on the Mäule-stained section were proven to be substantially variable between lines (**Fig. 2**). The number of cell layers in the cortical sclerenchyma varied from 2 to 4

Table 3 Genetic variation in maize stems for phenolic contents. Data from (1) Méchin *et al.* 2000, (2) and (3) INRA Lusignan unpublished results. ADL (acid detergent lignin) and KL (Klason lignin) as NDF % (neutral detergent fiber); pCA (*p*-coumaric acid) and FA (ferulic acid) as mg/g NDF; H, G, and S (*p*-Hydroxyphenyl, Guaiacyl, Syringyl) lignin units released after thioacidolysis, and Hn, Vn, Sn (*p*-Hydroxybenzaldehyde, Vanillin, Syringaldehyde) lignin units released after nitrobenzene oxidation as µmol/g KL. Analytical methods used in experiments 2 and 3 were the same as in experiment 1.

Genotype	ADL	KL	Ester pCA	Ester FA	Ether FA	Lignin subunits			
13 lines (1)						H	G	S	S/G
mini	4.97	14.7	17.3	-	1.41	10	301	430	1.03
maxi	9.55	19.9	27.8	-	2.32	30	591	632	1.79
mean	6.90	17.1	21.5	-	1.80	16	380	515	1.40
19 lines (2)						Hn	Vn	Sn	Sn/Vn
mini	3.14	10.6	8.1	5.33	0.99	76	290	256	0.94
maxi	4.82	14.1	11.5	6.06	1.26	109	451	380	1.23
mean	4.07	12.4	10.3	5.79	1.12	92	355	310	1.04
23 lines (3)									
mini	3.89	11.6	7.0	4.70	1.05	92	372	373	0.85
maxi	6.43	17.3	16.9	6.70	2.36	212	682	751	1.24
mean	5.49	15.4	12.0	5.66	1.35	157	530	558	1.05

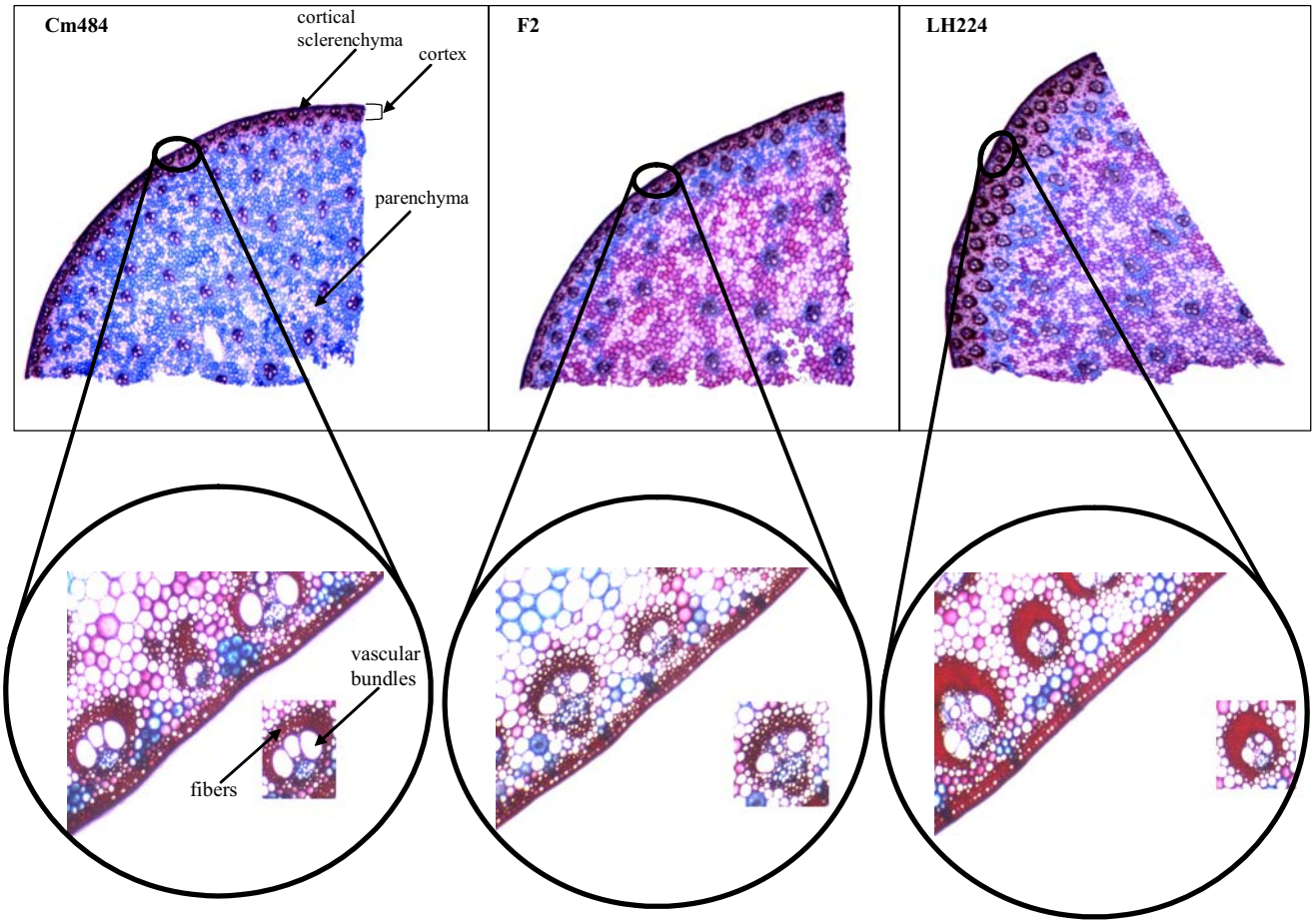

Fig. 2 Variation for lignification traits in stalk parenchyma and fibers of three maize inbred lines. Maize inbred lines Cm484, F2, and LH224 have increasing lignin contents and decreasing values of cell wall digestibility. Transversal histological slides in basal internode of mature maize plants show non-lignified and lignified areas which are Fasga-stained in blue and red, respectively. Parenchyma of Cm484 is not lignified, while is nearly completely lignified in F2. Vascular fibers of LH224 are strongly lignified, while their lignification is low in F2.

between inbred lines, with a variation in cell layer thickness in the cortical sclerenchyma ranging from 34 to 69µm. Furthermore, the lignified (red) area ranged from 1 to 5-fold higher in maize stalk cross section. Lines such as F2 and LH224 had thus a highly lignified parenchyma while others as Cm484 had a slightly, even non-lignified, parenchyma (**Fig. 2**). Lignification intensities in fibers surrounding vascular bundles were also greatly variable, very important in LH224 as compared to F2 or Cm484 (**Fig. 2**). Moreover, fiber lignification intensities were not related to those of parenchyma. These results strengthen the importance of the understanding of regulations of lignification in each tissue, which appeared (partly) independent towards the definition of maize and grass ideotypes.

VARIATION IN EXPRESSION OF MAIZE LIGNIN PATHWAY GENES

Gene expression variation in internodes according to their physiological maturity

Based on expression data of Guillaumie *et al*. (2007b), the expression of phenylpropanoid and related genes in young stems of 20-day-old plants is highly correlated with their expression in the older basal internodes of silking plants (r = 0.91). Conversely, gene expression in lignifying ear internodes of silking plants is more weakly related to their expression in basal internodes (r = 0.55), and to a still lower extent with their expression in young stems (r = 0.31). Several genes are not significantly expressed in young stems, or are no longer expressed in physiologically old basal internodes, whereas they are fully expressed in ear internode at silking stage. Moreover, some family members appear of weak importance in the lignin constitutive pathway, based on their level of expression.

An inventory of phenylpropanoid and related genes expressed in basal (physiologically old) and ear (physiologically young) internodes of silking plants (**Table 2**), have been conducted from data of Guillaumie *et al*. (2007b). Three PAL out of the four investigated genes appeared of importance in the maize lignin pathway. One PAL gene is at least equally expressed as the previously described MZEPAL gene (www.MaizeGDB.org). CCoAOMT5 and CCoAOMT2 appeared to be more specifically expressed in physiologically young internode, while CCoAOMT3 appeared to be more specifically expressed in physiologically old internode. CCoAOMT1 and CCoAOMT4 genes appeared to be of less importance than the other three genes in the constitutive lignin pathway. Only five out of the eight investigated CCR genes have a significant expression in basal internode, respectively, while all CCR genes were significantly expressed in the ear internode. Possibly due to the absence of data for other F5H genes, the F5H1 expression was much lower than the COMT expression in ear or basal internodes. Moreover, F5H1 and COMT expression was nearly four times higher in ear than in basal internode. In older basal internode, the ZmCAD1 gene was more expressed than the reference ZmCAD2/*bm1* gene. In ear internodes, the strongest expressed gene was as expected the ZmCAD2/*bm1* gene. However, the SAD gene, which is the closest ortholog to the aspen gene, and the ZmCAD1 gene were also greatly expressed. For the oxidative polymerization of monolignols, laccase genes appeared to be more expressed than peroxidase genes. Moreover, genes of these two families appeared to be more expressed in basal older internode than in younger ear internode, probably because they correspond to the

last step of cell wall lignification. All these gene expression results emphasize the importance of multigene families in the maize lignin pathway. Moreover, in each family, the most expressed gene(s) are not always those currently considered or described in the maize lignin pathway, and the weak expression of several key-genes strengthens the hypothesis that all members of several families have not been yet discovered.

Variation of lignin pathway gene expression in maize brown-midrib mutants

Expression studies of phenylpropanoid and related genes in young lignifying stems of *bm1*, *bm2*, *bm3*, and *bm4* plants (Guillaumie *et al*. 2007a) have given a complementary characterization of these mutants with original information on the lignin pathway.

In young stems of 20-day-old plants (Guillaumie *et al*. 2007a), the ZmCAD2 gene was, as expected, under-expressed in *bm1* plants in agreement with the previous observation of enzymatic activities by Halpin *et al*. (1998). However, all other investigated CAD and SAD genes were also under-expressed, simultaneously with several regulatory genes including MYB, AGO and HDZip genes. It was therefore considered that the *bm1* mutation occurs in a gene located in bin 5.04, close to the ZmCAD2 gene, which regulates the expression of several members of the CAD/SAD gene family. The involvement of an interfering RNA process causing the under-expression of the CAD genes in *bm1* plants was thus hypothesized, since the most under-expressed *AGO* gene (AY111249) in *bm1* plants was likely, from synteny data with rice, located in maize bin 5.04, nearly 1 cM upstream the ZmCAD2 gene.

In young stems of the *bm2* mutant, the maize gene ZmZAG5 (SHATTERPROOF MADS-box SHP1 ortholog) gene was under-expressed to nearly the same level as the disrupted COMT gene in *bm3* plants. Simultaneously, the AGO ortholog (AY104211) and the ATHB-8 HD-zip III ortholog (CO529337) were also greatly under-expressed. A lower expression of an AGO gene would induce a lower repression of target genes (Baumberger and Baulcombe 2005). The contrasting pattern of expression of ZmZAG5, AGO, and ATHB-8 HD-zip III regulation genes between *bm3* plants with low S content in lignins, and *bm2* plants with low G content in lignins is surely a significant clue in monomer regulation, or in patterning of S and G rich tissues. The SHP1 gene, together with SHP2, is considered to specify the lignified valve margin of mature *Arabidopsis* siliques (Liljegren *et al*. 2000) and both AGO and ATHB-8 HD-zip III genes are involved in miRNA regulation. This simultaneous modified expression of *AGO* and *HD-Zip III* genes in *bm2* plants strengthen the hypothesis of a *bm2* mutation affecting a gene involved in the RNAi silencing complex in a collaborative action with the maize ZmZAG5 gene, which is not yet understood. It should have consequences on the formation of G-rich tissue and/or possibly the xylem/phloem ratio, which could be related to the tissue-specific lignification disturbance observed in *bm2* mutants (Vermerris and Boon 2001).

The pattern of gene expression in *bm4* young stems (Guillaumie *et al*. 2007a) resembles in part those observed in *bm1* and *bm2* mutants, but with a lower number of under-expressed genes that were less under-expressed than in *bm1* and *bm2* plants. Moreover, the *bm4* mutant is characterized by the over-expression of phenylpropanoid and methylation genes. As it was considered from biochemical data, the *bm4* mutation is poorly understood even after these expression studies. It can be hypothesized the *bm4* mutation affects a gene involved in the regulation of lignification or lignified tissue patterning.

Expression studies of phenylpropanoid and related genes in young lignifying stems of *bm3* plants (Guillaumie

Table 4 Expression of several O-methyltransferase and regulation genes in ear internode of normal and *bm3* INRA F2 isogenic plants at silking stage. Normalized expression values are given for the F2 line, with the F2*bm3*/F2 ratio of signal intensities. Results are based on data of Guillaumie (2006), expression values higher than 2.0 or lower than 0.5 are significantly higher or lower than reference values, respectively.

Genes	mRNA	Gene expression	
		F2	F2*bm3*/F2
Caffeic acid O-methyltransferase (COMT *bm3*)	M73235	142203	0.05
Caffeoyl CoA O-methyltransferase (CCoAOMT1)	AJ242980	21976	0.88
Caffeoyl CoA O-methyltransferase (CCoAOMT2)	AJ242981	47434	0.77
Caffeoyl CoA O-methyltransferase (CCoAOMT3)	AY104670	23740	3.18
Caffeoyl CoA O-methyltransferase (CCoAOMT4)	AI855419	13947	2.38
Caffeoyl CoA O-methyltransferase (CCoAOMT5)	AY108449	51550	1.01
O-methyltransferase (OMT ZRP4 ortholog)	AY105091	14318	1.70
O-methyltransferase (OMT ZRP4 ortholog)	AY108765	26598	1.71
ARGONAUTE	CD441197	13525	3.64
ATHB-8 HD-zip III	CO529337	14535	2.88
ZmZAG5 (SHATTERPROOF MADS-box SHP1)	L46398	15008	3.50

Table 5 Expression variation of lignin pathway genes in the two F268 and F286 maize lines at silking stage. F268 and F286 differed in lignin content (6.4 and 5.2% ADL/NDF, respectively, NDF and ADL according Goering and van Soest 1970) and cell wall enzymatic degradability (22 and 32% NDF degradability, respectively, NDF enzymatic degradability according to Struik 1983 and Dolstra and Medema 1991). Normalized expression values are given for the F268 line, with the F286/F268 ratio of signal intensities. Unpublished data of INRA Lusignan and UMR5546 Toulouse obtained with same methods as those used by Guillaumie *et al*. (2007a), expression values higher than 2.0 or lower than 0.5 are significantly higher or lower than reference values, respectively.

Genes of the lignin pathway	mRNA	Expression in silking plants	
		F268	F286 / F268
Phenylalanine / Tyrosine ammonia lyase	AC185453	57310	0.31
Phenylalanine / Tyrosine ammonia lyase (MZEPAL)	AY103947	31307	0.70
Phenylalanine / Tyrosine ammonia lyase	AY106831	116420	0.26
4 Coumarate CoA ligase (4CL1)	AY105108	14672	0.42
Caffeoyl CoA O-methyltransferase (CCoAOMT1)	AJ242980	9656	0.77
Caffeoyl CoA O-methyltransferase (CCoAOMT2)	AJ242981	13612	0.74
Caffeoyl CoA O-methyltransferase (CCoAOMT3)	AY104670	18453	0.82
Caffeoyl CoA O-methyltransferase (CCoAOMT4)	AI855419	7923	0.79
Caffeoyl CoA O-methyltransferase (CCoAOMT5)	AY108449	71160	0.39
Caffeic acid O-methyltransferase (COMT)	M73235	81210	0.36
Cinnamyl alcohol dehydrogenase (ZmCAD2)	Y13733	42403	0.35

et al. 2007a) were strengthened with a study in silking *bm3* plants (Guillaumie 2006). As expected the disrupted COMT was greatly under-expressed in *bm3* plants, similar to a null expression, with activation of alternative methylation pathways. In young stems, two O-methyl transferase (OMT) and two cytochrome P450 genes were significantly over-expressed. In the ear internode of silking plants, CCoAOMT3 and CCoAOMT4 genes were highly over-expressed despite they are considered to have a low affinity towards 5-hydroxy-coniferaldehyde (**Table 4**). Complementarily, two other

Table 6 Putative QTL for lignin content observed in eight recombinant inbred line progenies in *per se* value experiments. Data from (1) 100 RIL F2 × Io (Méchin *et al.* 2001), (2) 131 RIL F288 × F271 (Roussel *et al.* 2002), (3) 242 RILs F838 × F286 (INRA - ProMaïs unpublished data), (4) 186 RIL B73 × B52 (Cardinal *et al.* 2003b), (5) 200 RIL B73 × De811 (Krakowsky *et al.* 2005; Krakowsky, com. pers.), (6) 140 RIL Fl1 × F2 (INRA - Biogemma unpublished data), (7) 231 RIL F7025 × F4 (INRA - Biogemma unpublished data), (8) 164 RIL FR × WM13 (INRA - RAGT/R2n unpublished data). ADL is acid detergent lignin according to Goering and van Soest (1970) and is expressed as % DM (dry-matter, RIL 4, 5) or % NDF (neutral detergent fiber, RIL 1, 2, 3, 6, 7, 8). QTL positions and support intervals are given as centimorgans (cM) on each chromosome. Maize bins are successive areas nearly equal to 20 cM separated by RFLP markers on each chromosome. Lod values indicate how much probable is the presence than the absence of the QTL, according to Lander and Botstein (1989). R^2 is the percentage of ADL lignin phenotypic variation explained by the QTL and line (+) increased the ADL lignin content. Analytical methods used in experiments 3, 6, 7, and 8 were the same as in experiment 2.

RIL progeny	RIL reference	Chr-pos	Bin	Closest marker	Support interval	lod	R^2	Line (+)
Fl1 × F2	6	1-60	1.02	umc1976	52-72	3.8	12	F2
F838 × F286	3	1-80	1.02	bnlg1178	66-92	3.5	6	F838
F7025 × F4	7	1-76	1.03	bnlg176	68-86	9.5	17	F7025
F838 × F286	3	1-174	1.05/06	bnlg1832	148-190	4.2	8	F838
FR × WM13	8	1-112	1.06	umc1838	106-118	5.0	13	FR
B73 × B52	4	1-124	1.07	bnlg615	114-128	5.6	16	B52
B73 × De811	5	1-151	1.07	umc33a	145-160	4.7	17	De811
B73 × B52	4	1-179	1.10	isu106a	160-186	3.5	5	B52
Fl1 × F2	6	2-2	2.00	umc2246	0-10	3.6	13	Fl1
F7025 × F4	7	2-102	2.02	bnlg2277	78-110	3.1	6	F7025
FR × WM13	8	2-72	2.06	bnlg1831	66-80	8.5	21	WM13
F7025 × F4	7	2-156	2.07	bnlg1045	134-168	3.0	6	F7025
B73 × B52	4	2-110	2.08	umc4	100-118	5.3	6	B52
B73 × De811	5	2-112	2.08	phi127	105-115	7.4	14	De811
F838 × F286	3	2-192	2.09	bnlg1520	188-192	5.5	10	F286
Fl1 × F2	6	3-2	3.01	umc1746	0-8	4.0	13	F2
Fl1 × F2	6	3-22	3.01	umc2377	18-30	5.2	16	Fl1
B73 × B52	4	3-24	3.01	umc121a	12-38	3.4	7	B73
B73 × De811	5	3-39	3.02	php20042	20-50	3.0	6	De811
FR × WM13	8	3-90	3.04	bnlg1505	70-104	6.2	16	WM13
B73 × B52	4	3-62	3.04/05	dupssr5	60-73	11.7	17	B52
F288 × F271	2	3-140	3.05/06	bnlg1505	132-150	2.6	11	F271
FR × WM13	8	3-120	3.08	umc2048	116-134	3.4	9	FR
FR × WM13	8	3-164	3.08	umc1320	154-166	3.0	8	FR
Io × F2	1	4-92	4.05	sc315s	82-104	2.4	10	Io
F7025 × F4	7	4-72	4.06	bnlg252	64-80	7.3	14	F4
F7025 × F4	7	4-128	4.08	bnlg2162	124-142	3.2	6	F4
FR × WM13	8	4-114	4.08	dupssr28	104-122	3.1	9	FR
B73 × B52	4	4-182	4.10	bnlg589	170-192	3.0	4	B73
F7025 × F4	7	5-58	5.03	bnlg1046	38-74	2.8	5	F4
B73 × De811	5	5-59	5.03	bnl5.02	55-65	5.4	12	De811
B73 × B52	4	5-62	5.03	bnl7.43	57-63	4.1	11	B52
Fl1 × F2	6	5-104	5.03	umc2296	94-112	5.0	16	F2
B73 × De811	5	5-83	5.04	bnl7.71	70-95	3.4	7	B73
FR × WM13	8	5-90	5.05	umc1482	72-102	3.7	10	WM13
B73 × B52	4	5-116	5.06	phi087	114-120	3.2	4	B73
B73 × De811	5	5-136	5.07	phi128	125-140	3.5	16	B73
F288 × F271	2	6-20	6.01	bnlg1867	10-24	2.0	7	F271
Fl1 × F2	6	6-22	6.01	phi077	18-30	3.9	12	F2
F7025 × F4	7	6-92	6.04	umc1857	80-102	5.2	10	F7025
B73 × B52	4	6-70	6.05	umc21	60-74	4.0	11	B52
B73 × De811	5	6-71	6.05	bnl5.47	65-85	11.3	17	De811
F288 × F271	2	6-184	6.06	bnlg345	162-194	6.5	20	F271
B73 × De811	5	6-116	6.07	phi123	105-125	3.1	6	De811
F838 × F286	3	7-6	7.00	bnlg2132	0-20	3.3	6	F286
B73 × De811	5	7-147	7.06	umc168	140-150	3.5	6	B73
B73 × B52	4	8-64	8.03	phi081	62-68	4.2	4	B52
F7025 × F4	7	8-88	8.05/06	bnlg1782	78-96	7.3	14	F4
B73 × B52	4	8-156	8.08	phi080	148-164	2.0	4	B52
F838 × F286	3	8-156	8.09	umc1638	136-172	3.4	6	F838
F288 × F271	2	9-110	9.02	bnlg1401	98-122	3.3	11	F288
Fl1 × F2	6	9-86	9.05	bnlg1270	74-100	3.1	10	Fl1
B73 × De811	5	9-96	9.06	npi209	90-100	2.6	6	B73
F7025 × F4	7	9-124	9.06	bnlg1191	122-134	6.3	12	F7025
F838 × F286	3	10-32	10.02	umc1337	24-40	5.7	10	F286
Fl1 × F2	6	10-58	10.03	bnlg1079	54-66	3.1	10	F2
B73 × B52	4	10-110	10.05	npi232	96-116	8.8	13	B73
FR × WM13	8	10-102	10.06	bnlg2190	94-108	6.3	17	FR

OMT genes were slightly over-expressed and could also be considered as active on 5-hydroxy-coniferaldehyde. No transcription factors were deregulated in young *bm3* stems, while several transcription factors or regulation genes were deregulated in the ear internode of *bm3* silking plants. More especially, the ZmZAG5 ortholog to *Arabidopsis* SHATTERPROOF MADS-box SHP1, the ARGONAUTE (AGO) ortholog (AY104211), and the ATHB-8 HD-zip III ortholog (CO529337) were three times or more over-expressed (**Table 4**).

Variation of lignin pathway gene expression in differentially lignified maize lines

Previous results have strengthened the effect of physiological tissue maturity and *bm* mutations on gene expression in the lignin pathway. Complementarily, genotype effects on gene expression were recently investigated in ear internode of silking plants of two flint lines with different lignin content and cell wall enzymatic degradability (5.2 and 6.4% ADL/NDF, and 32 and 22% IVNDFD, in F286 and F268, respectively; ADL and NDF are Acid Detergent Lignin and Neutral Detergent Fiber according Goering and van Soest 1970; IVNDFD is *in vitro* NDF digestibility according to Struik (1983) and Dolstra and Medema 1990). Several genes of the lignin pathway appeared under-expressed in the line which has the lower lignin content (F286). Especially two PAL, CCoAOMT5, COMT, and ZmCAD2 genes had a 3-fold reduced expression level in F286, while the 4CL1 was half expressed (**Table 5**). Shi *et al*. (2007) have also observed a lower expression of the ZmCAD2 and PAL genes in two sets of RIL differing for their cell wall digestibility. Several genes of the lignin pathway and their regulation factors might thus be relevant target for breeding grasses with modified lignin contents, after these findings have been validated in other genetic backgrounds.

QTL ANALYSES FOR PHENYLPROPANOID CONTENTS IN GRASS CELL WALLS

Genetic variation for traits related to lignification has allowed the development of different RIL progenies and determination of genomic locations involved in lignin traits. QTL for lignin content in maize are available at least from data in eight RIL progenies (**Table 6**). Based on ADL/NDF (or ADL in dry-matter) content measurements, 58 QTL have been found, 26 of which explained at least 10% of the phenotypic variation for lignin content in different RIL progenies. A meta-analysis was investigated based on a projection of all maps and QTL on the reference map IBM2 Neighbors (www.MaizeGDB.org) with the Biomercator software (Arcade *et al*. 2004), followed by a restriction of markers to those of the reference map IBM_GNP2004 map (Falque *et al*. 2005). After projection, the 58 individually observed QTL corresponded to no more than 43 different locations (**Fig. 3**). QTL were scattered throughout the maize genome, except on chromosome 7 which carried only two QTL of low R^2 values. Several QTL were found in isolated locations although they have high R^2 values in individual progenies. An important hot-spot was observed in bin 5.03 (/5.04) in the upstream region of the *bm1* location with a cluster of five QTL. Similar colocalizations gathering three QTL were observed in bin 2.08, 6.04 and 9.06, respectively. Two QTL colocalized exactly in bin 1.07, with alleles increasing lignin content that originate from the corn borer resistant lines B52 and DE811. In bin 4.05, two QTL were found in a similar location close to the *bm3* position. Other colocalizations of two QTL were observed in bins 3.01, 4.08, 6.06, and 8.08, respectively.

No QTL has been described to date for H, G, and S lignin units in grasses, except one QTL for S/G ratio given by Ni *et al*. (1998) in bin 7.02. Only two investigations (Ni *et al*. 1998; Fontaine *et al*. 2003b) are available giving QTL positions for pCA and FA content in grasses (**Table 7**). Among the five QTL for pCA content, two were located in bins 7.02 and 9.01, respectively, in which no QTL for lignin content were found in any of the eight available RIL progenies. Similarly, only one QTL for esterified FA content out of three was located in a location (bin 4.08) in which a QTL for lignin content has also been found. QTL for etherified FA content have been detected in four locations (Ni *et al*. 1998; INRA Lusignan unpublished data). In rice, three QTL for FA content were found by Dong *et al*. (2005), based on an analytical method measuring simultaneously free and cell-wall-linked ferulate. Two QTL out of three explained more than 10% of the phenotypic variation. The first QTL was located on the upper part of the rice short arm of chromosome 3, which is highly collinear with the short arm of maize chromosome 1 and the inverted long arm of maize chromosome 9 (The Rice Chromosome 3 Sequencing Consortium 2006). This rice QTL could thus be orthologous to the one found by Ni *et al*. (1998) close to the *bm4* gene which has been mapped in bin 9.06/08, at the bottom of chromosome 9.

Putative candidate genes are available for several locations but most candidate genes have not been validated. For several other QTL, relevant candidate genes have yet to be defined. Whereas brown-midrib mutations were proven to affect lignin content and structure, no QTL were observed in bins 1.11 and 9.06/08, which contain the *bm2* and *bm4* genes, and only one in the same position as the *bm1* gene. Complementarily, only two QTL were observed in bin 4.05, a result which is somewhat contradictory with the great impact of the *bm3* mutation and COMT down-regulation on lignin content (Piquemal *et al*. 2002; He *et al*. 2003), or with the lower expression of COMT gene in lower lignified lines. It could thus be considered that the expression of the COMT gene is regulated by transcription factors located elsewhere in the genome and likely in one of the other locations supporting lignin QTL. It could be also supposed that the under-expression of the COMT gene is related to down-regulation of the whole pathway with a coordinate regulation of at least PAL, CCoAOMT and COMT (**Table 5**). PAL homologs are mapped in bin 5.05, 2.03, and 4.05 (pal1, pal2, pal3 or bnl17.23, bnl17.23b, and bnl17.23c, respectively, www.MaizeGDB.org), in locations which are similarly not QTL-rich. The map position of all CCoAOMT genes is not publicly available. However, these results strengthen the importance of regulatory factors underlying QTL of lignin content rather than genes directly involved in the monolignol biosynthesis. In agreement with these findings, eQTL (expression QTL) of genes putatively involved in cell wall digestibility and including lignin pathway genes were investigated by Shi *et al*. (2007). Five hotspots containing more than 5% to more than 20% of the total number of eQTL were found in bins 1.12, 9.04, 1.07, 3.05, and 8.03, respectively, which did not correspond with QTL locations important for lignin content.

BREEDING GRASSES WITH OPTIMAL CELL WALL PROPERTIES

Towards a new ideotype of forage and biofuel grass

For both animal feeding and green energy production, breeding schemes only focused on cell wall traits are insufficient, as grasses of the future must be plants capable of growing in sustainable cropping conditions. The most relevant breeding objective is therefore to increase the yield of degradable DM, with as little as possible nitrogen and water needs, while preserving the agronomic improvements that have been achieved in yield stability across environments, stalk standability, disease resistance, grain filling rate, and stay-green performances. Due to climatic changes and global warming, breeding for an improved capability to endure and recover after hot and dry conditions is an inescapable objective for grasses of the future, with improved cell wall degradability.

To date, most studies related to cell wall degradability

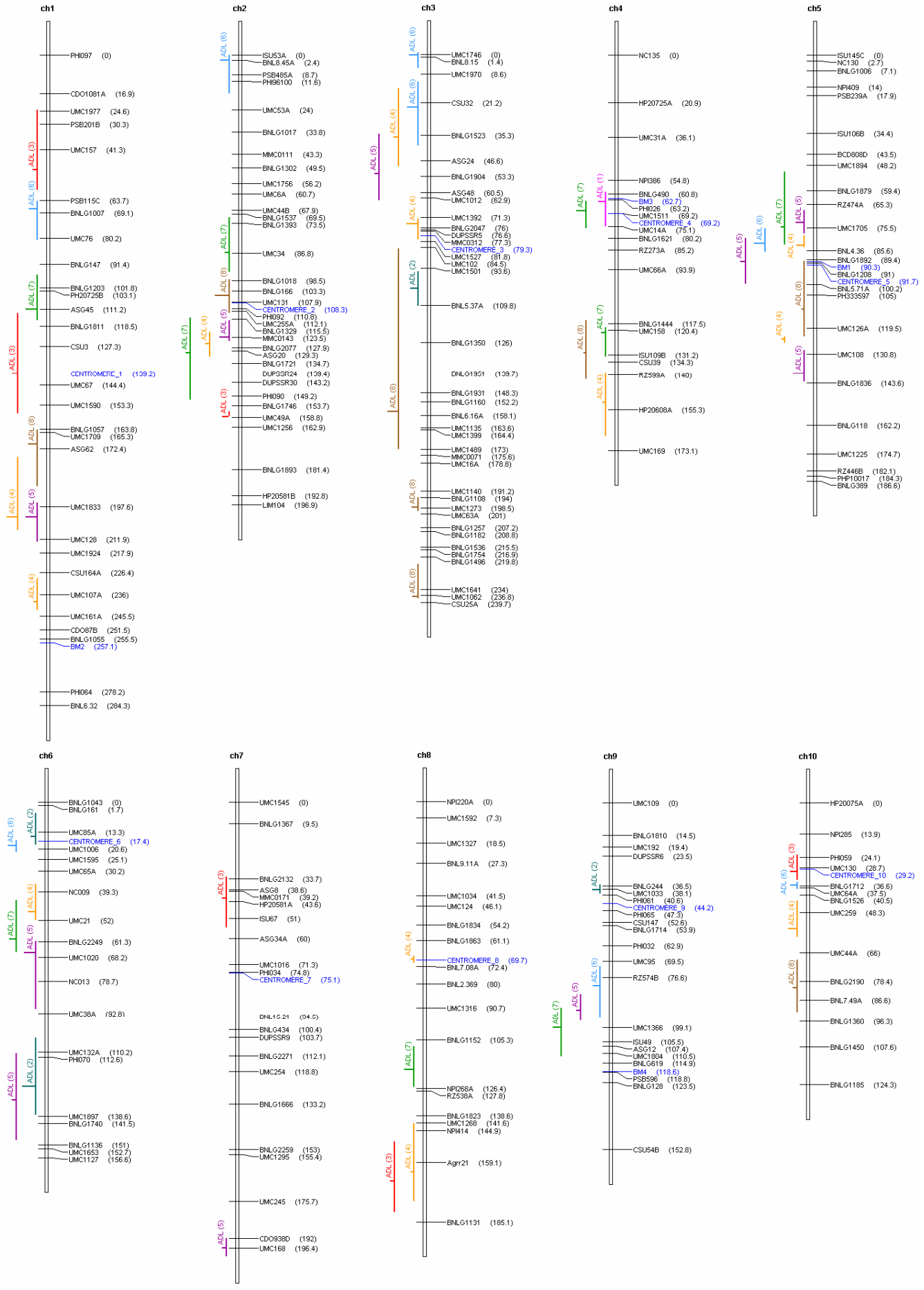

Fig. 3 QTL mapping for lignin content after a meta-analysis from eight maize RIL progenies. Lignin is expressed as acid detergent lignin (ADL, Goering and van Soest 1970) and cell wall is expressed as neutral detergent fiber (NDF, Goering and van Soest 1970). In bracket number of each QTL corresponds to each RIL progeny according to the original data given in **Table 6**.

Table 7 QTL for hydroxycinnamic acid content in maize stem and rice straw. Maize data from (1) 45 RIL Cm37 x T232 (Ni *et al*. 1998) and (2) 131 RIL F288 × F271 (Fontaine *et al*. 2003b). Rice data from (3) 165 RIL Asominori × IR24 (Dong *et al*. 2005). Maize ester pCA and ester FA are esterified *p*-coumaric and ferulic acids, respectively, and ether Fa is etherified ferulic acid, all released after alkaline hydrolysis according to Morrison III *et al.* (1993). Rice esterified ferulic acid included free ferulic acid. QTL positions and support intervals are given as centimorgans (cM) on each chromosome. Maize bins are successive areas nearly equal to 20 cM separated by RFLP markers on each chromosome. Lod values indicate how much probable is the presence than the absence of the QTL, according to Lander and Botstein (1989). R^2 is the percentage of trait phenotypic variation explained by the QTL and line (+) increased trait values. - = missing data.

	RIL progeny	chr-pos	bin	Closest marker	Support interval	Lod	R^2	Line (+)
Maize								
ester pCA	2	3-238	3.08	umc1273	218-254	2.2	7.5	F288
ester pCA	2	4-220	4.09/10	umc1109	210-230	2.8	9.2	F271
ester pCA	2	6-78	6.04	bnlg1617	70-100	3.1	10.3	F288
ester pCA	1	-	7.02	npi568	-	-	-	-
ester pCA	2	9-14	9.01	mc2122	10-24	2.6	8.6	F288
ester FA	2	2-72	2.04	mc1175	64-78	2.4	8.1	F288
ester FA	2	3-74	3.03/04	bnlg1452	52-108	2.3	7.6	F271
ester FA	2	4-110	4.08	umc1418	98-116	2.6	8.7	F288
ester FA	1	-	7.02	npi568	-	-	-	-
ether FA	2	1-220	1.06/07	bnlg1025	190-226	1.6	6.0	F288
ether FA	1	-	1.11	*bm2*	-	-	-	-
ether FA	1	-	4.05	*bm3*	-	-	-	-
ether FA	1	-	9.06/08	*bm4*	-	-	-	-
Rice								
ester FA	3	3-2	-	G1318	0-3	2.0	5.5	Asominori
ester FA	3	6-47	-	C574	45-49	3.2	16.9	Asominori
ester FA	3	7-94	-	XNpb379	94-107	3.1	12.8	Asominori

improvement have been based either on lignin content or on a global cell wall enzymatic solubility trait such as IVNDFD or DINAGZ (*in vitro* digestibility of the cell wall according to Barrière *et al*. 2003). Variations in lignin content are not sufficient to explain variations in cell wall degradability. Therefore, an efficient breeding for increased cell wall degradability necessitates the dissection of this trait in each of its genetic underlying components. Esterified pCA and etherified FA contents explained a significant part of the phenotypic variation in enzymatic cell wall digestibility, and an increasing proportion of ferulate dimers reduced carbohydrate release after enzymatic hydrolysis (Grabber *et al*. 1998). How etherified ferulate reflect total ferulate and particularly diferulate cross-linking is not known (Grabber *et al*. 2004), but breeding for a reduced level of ether-linked ferulate has improved bromegrass cell wall degradation by rumen microorganisms (Casler and Jung 1999). Attempts to understand the impact of lignin structure, commonly described by the S/G ratio, on the cell wall susceptibility to enzymatic hydrolysis led to conflicting results (Grabber *et al*. 1997; Méchin *et al*. 2000). However, together with high lignin content, an increased proportion of H units contributed to the hardening of maize endodermis cell walls (Degenhardt and Gimmler 2000; INRA Lusignan and Génoplante unpublished data).

The future availability of highly degradable maize (or any grass), either for animal feeding or biofuel production, will require a new investigation of genetic resources including those that are not currently used, or that were never used in plant breeding. Use of specific genetic resources is all the more necessary as an average decline in maize plant digestibility between 1955 and 2005 was shown to be related to a change in germplasm for both dent and flint elite parental lines, mostly bred for grain production (Barrière *et al*. 2004a, 2005). However, most of improvements progressively piled up in modern (grain) lines and hybrids should be preserved when breeding of genotypes for high energy value.

Adverse indirect correlated responses when breeding maize and grass for increased cell wall degradability

Lignins, which contribute with ferulate cross-linkages to stalk strength and rigidity and impede cell wall degradability, are also involved in disease and pest resistance. Several *bm3* lines were thus more susceptible to *Ustilago maydis* smut than their normal counterparts (INRA Lusignan unpublished data). Similarly, the lignin content of maize stalks has been associated with resistance to stalk-tunneling by the European corn borer (ECB, *Ostrinia nubilalis* Hübner) and correlated responses in ECB feeding on stalks were observed after one cycle of divergent selection for lignin content in this tissue in some populations (Coors 1987; Beeghly *et al*. 1997; Ostrander and Coors 1997). DIMBOA (2,4-dihydroxy-7-methoxy-1,4-benzoxasin-3-one), which occurs as a glucoside in intact tissue, has for a long time been known to be involved in maize leaf feeding resistance to the ECB first-generation (Klun *et al*. 1967). Other mechanisms by which increases in cell wall components (including lignins) concentrations cause reduced ECB tunneling have been proposed. An increase of fiber and lignin concentration may increase the bulk density in the ECB diet, reduce nutrients and available energy, reduce ECB larvae growth, and consequently reduce the tunneling by this insect (Rojanaridpiched *et al*. 1984; Bernays 1986; Coors 1987; Buendgen *et al*. 1990; Beeghly *et al*. 1997). Leaf toughness caused by high concentration of cell wall components is associated with resistance to ECB feeding (Bergvinson *et al*. 1994). Moreover, leaf and stalk stiffness caused by cross-linkages between cell wall components (Grabber *et al*. 1995, 2000a; MacAdam and Grabber 2002; Barrière *et al*. 2004b, 2005) is likely also a trait involved in ECB tolerance.

QTL involved in stalk tunneling length and/or damage rating under infestation of European corn borer have been investigated in six studies, corresponding to nearly 48 bin locations (Schon *et al*. 1993; Bohn *et al*. 2000; Papst *et al*. 2001; Cardinal *et al*. 2001; Jampatong *et al*. 2002; Cardinal *et al*. 2003a; Krakowsky *et al*. 2004). In the B73 × B52 and B73 × De811 RIL progenies, three QTL for ECB tunneling were associated with QTL for cell wall components content in a manner expected for pleiotropic mechanisms (Cardinal *et al*. 2003b; Krakowsky *et al*. 2004; Cardinal and Lee 2005). Moreover, based on lignin QTL meta-analysis and ECB data, colocalizations of QTL for corn borer resistance and lignin content are expected primarily in bins 1.07, 2.08, 5.03, 5.05, 6.07. Although none of the QTL detected for *in vitro* digestible organic matter of stover colocalized with QTL for ECB tunneling/damage in the study of Papst *et al*. (2001), several of the QTL involved in corn borer resistance correspond to locations also involved in cell wall digestibility (Ralph *et al*. 2004a). However, QTL colocalization for

different traits is not proof of common genetic mechanisms, but it cannot be dismissed that cell wall content, lignin content and composition, and tissue stiffness are traits involved in ECB tolerance. Genotypes with high cell wall digestibility could therefore be more susceptible to pest damages and alternative genetic mechanisms for insect resistance may be needed for the improvement of forage or biofuel maize. Therefore, corn borer susceptibility should be estimated simultaneously with lignin and cell wall digestibility traits, and be used as one selection criteria in forage or biofuel maize improvement programs.

Functional markers in breeding for higher cell wall digestibility

Efficient breeding for quantitative or complex traits such as cell wall qualities can be improved by the use of marker assisted selection (MAS). As soon as a QTL has been detected and validated, its marker assisted introgression based on the two flanking markers into an elite genetic background is possible. The major drawback of anonymous genetic markers (isozymes, or random DNA markers such as microsatellites, AFLP, RFLP, etc.) is that their predictive value depends on the known linkage phase between markers and target locus alleles (Lübberstedt *et al*. 1998). Thus, (quantitative) trait locus mapping is necessary for each cross *de novo*, as different subsets of QTL are polymorphic in individual populations, and linkage phases between marker and QTL alleles can disagree even in closely related genotypes.

In contrast to anonymous genetic markers and according to Andersen and Lübberstedt (2003), functional markers (FM) are derived from polymorphic sites within genes causally involved in phenotypic trait variation. Once genetic effects have been assigned to functional sequence motifs, FM derived from such motifs can be used for fixation of gene alleles (defined by one or several FM alleles) in a number of genetic backgrounds without additional calibration. FM development requires first functionally characterized genes and allele sequences from such genes. Secondly, polymorphic and functional motifs affecting plant phenotype within these genes must be identified followed by a validation of associations between DNA polymorphisms and trait variation in a large set of germplasm. However, in heterogeneous genotype collections, associations identified for specific sites might be confounded with effects from other genome regions especially in case of population stratification (Pritchard *et al*. 2000), which needs to be taken into account for interpretation of results from association studies. Finally, application of FM also depends on the availability of robust marker assay technologies.

Whereas assignment of an "agronomic function" to short sequence motifs can be achieved by candidate gene based association studies (Risch 2000), this approach is limited by linkage disequilibrium (LD), i.e. haplotype structures in the gene(s) of interest. However, for several genes, a generally low LD was detected in maize (Remington *et al*. 2001; Flint-Garcia *et al*. 2003), including examples in elite materials (Zein *et al*. 2007). Thus, candidate gene-based association studies are promising in maize. Varying levels of LD have previously been observed between genes of the phenylpropanoid pathway, decaying within few hundred bps for CCoAOMT2 and COMT (Guillet-Claude *et al*. 2004a; Zein *et al*. 2007) while spanning more than 3.5 kb at the PAL locus (Andersen *et al*. 2007).

Several candidate genes in relation to lignification (and forage quality) are meanwhile available from maize (Guillaumie *et al*. 2007b), or *Arabidopsis* (Costa *et al*. 2003; Goujon *et al*. 2003; Raes *et al*. 2003) investigations. The next step towards development of functional markers in maize breeding for cell wall quality traits is the identification of polymorphisms within these genes causally affecting the target trait. First reports on association studies for genes involved in cell wall biosynthesis confirm that genes of this pathway are promising targets for identification of polymorphic sites associated with cell wall quality, and thus for FM development. Zein *et al*. (2007) investigated the sequence variation at the COMT/*bm3* locus in a collection of 42 European maize inbred lines, variable for their stover IVNDFD and representative of elite germplasm for maize breeding in Central Europe. For association studies, stover digestibility has been investigated in six environments between 2001 and 2003 in Germany (heritability higher than 0.9). One INDEL polymorphism within the intron revealed significant association with stover digestibility (Lübberstedt *et al*. 2005). Complementarily, in a study of Guillet-Claude *et al*. (2004a), polymorphisms both in the COMT/*bm3* and the CCoAOMT2 coding genes showed significant association with maize digestibility. Such an association was not observed for the CCoAOMT1 gene, in agreement with expression results showing that this gene is weakly involved in the constitutive lignin pathway. A PAL gene was investigated in a set of 32 European elite inbred lines (Andersen *et al*. 2007). A one-bp deletion in the second exon of PAL, introducing a premature stop codon, was associated with a higher plant digestibility. Similarly, a polymorphism in the maize peroxidase gene ZmPox3, based on an insertion of a MITE also inducing a premature stop codon, was significantly associated with maize digestibility (Guillet-Claude *et al*. 2004b). Increasing availability of candidate gene sequences, and especially allele sequences for these candidate genes such as for the COMT, CCoAOMT2, CCoAOMT5, SAMS, ZmZAG5, …, will allow development of cost-efficient multiplexed single nucleotide polymorphism (SNP) marker assays. Whereas "traditional" DNA markers are assayed one by one or at low multiplex scale, SNP technology, driven by technological advance in human diagnostics, allows meanwhile parallel assessment of more than 1000 markers simultaneously. A recent example in higher plants is the establishment of a 1000-loci transcript map in barley (Stein *et al*. 2007), by using the Illumina Golden Gate Assay. Several other established SNP detection technologies provide sufficient flexibility, as required in plant breeding, to identify the economically optimal method for screening thousands of genotypes with a low or intermediate number of markers versus screening a moderate number of genotypes at high marker density. In conclusion, marker-assisted selection can be foreseen to become cheaper, while markers derived from candidate genes become increasingly more informative. As a tentative conclusion, availability of qualified candidate genes in the lignin pathway can be effectively converted into informative molecular markers by means of association studies. More can be expected with the discovery of genes involved in lignified tissue patterning and in regulation of lignification in each of these tissues.

CONCLUSIONS

Grass lignins are characterized by the specific amounts of H, G, and S units in the polymers, the importance of acylation of S units by p*CA*, and the importance of cross-linkage of G lignin units and arabinoxylans via FA bridges. Genes involved in the monolignol biosynthesis are known, while those involved in FA biosynthesis are to date partly hypothetic. However, the involvement of particular members within multigene families in each specific tissue and /or in each step of the biosynthesis remains for each of monolignols and *p*-hydroxycinnamic acids to be discovered. Specific channels for the biosynthesis of H, G, S, pCA and FA components, rooted more or less early in the shikimate or lignin pathways, correspond to a probable hypothesize with different members of multigene families and different groups of regulation factors involved in each route.

When taking the extent of cell wall polymer diversity (phenolics, carbohydrates, proteins) into consideration, the complex regulations that take place during growth towards establishment of tissue patterning, dynamic changes that occur during development and maturation of a given cell wall, the high degree of cell wall specificities in different cell types, it can considered that several hundreds or even thousands of genes are required for proper and coordinated

cell wall formation. QTL investigations have shown that at least 40 different loci are involved in controlling lignin content of maize, and likely ten or more have to be added if considering lignin monomeric composition and hydroxycinnamic acid content. According to publicly available and private data, several genes of the lignin pathway are mapped in locations corresponding to those where lignin content QTL were found. However, unknown genes, or genes with unknown functions, but involved in regulation of lignin pathway genes are likely more determinant factors of cell wall lignification traits (an subsequently of degradability variation), than variation in efficiency of structural genes of the lignin pathway. This has to be considered for the development of functional markers or the choice of target genes in mutant or transgenic approaches. Similarly genes involved in regulation of modes and duration of cell wall growth, tissue patterning, and cross-linkage of components are additional key-factors in understanding lignification in grasses.

Gene mining should be based on cell wall-related genomic and proteomic data sets which are now available in herbaceous model species such as *Arabidopsis* (Zhao *et al*. 2005; Jamet *et al*. 2006), zinnia (Milioni *et al*. 2001; Demura *et al*. 2002; Pesquet *et al*. 2005b, 2006), and also woody species including poplar (Hertzberg *et al*. 2001), pine and spruce (Pavy *et al*. 2005a, 2005b). For monocot species, resources and databases devoted to cell wall biosynthesis and assembly are emerging. A high throughput, FTIR-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://cellwall.genomics.purdue.edu). A bio-informatic and gene expression database of cell wall genes in maize has been published by Guillaumie *et al*. (2007b). Complementarily, a source of NMR data of lignin model compounds devoted to grasses has been designed at the USDA Forage Center (Ralph *et al*. 2004b). The search of genes specifically expressed in monocot based on a comparative bio-analysis approach has also been proven efficient (Mitchell and Shewry 2007) and should be developed between maize, sorghum, sugarcane and rice against *Arabidopsis* and other herbaceous or woody dicotyledons.

Genetic resources are available for an efficient short and possibly medium term cell wall degradability improvement of flint and dent lines even if most of modern elite dent lines have low cell wall digestibility. However, studies to understand the genetic and biochemical basis of cell wall qualities are a prerequisite for medium and long term improvements of forage and biofuel production.

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