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TOWARD THE DISCOVERY OF MAIZE CELL WALL GENES INVOLVED IN SILAGE QUALITY AND CAPACITY TO BIOFUEL PRODUCTION

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ABSTRACT - Silage maize is currently, with grazing, the basis of cattle feeding. In the near future, maize stover and cereal straws will also be a major source of carbohydrates for sustainable biofuel production. The embedding of cell wall in lignins and the linkages between lignins, *p*-hydroxycinnamic acids, and arabinoxylans greatly influences cell wall properties, including the enzymatic degradability of structural polysaccharides in animal rumen or industrial fermenters. Breeding for higher silage quality and biofuel production will thus be based on the discovery of genetic traits involved in each component biosynthesis and deposition in each lignified tissue. Genes involved or putatively involved in the biosynthesis of the grass cell wall were searched for, including phenolic compounds, cell wall carbohydrates and regulation factors. While most cytosolic steps of monolignol biosynthesis have been identified, most of lignin pathway genes belong to small multigene families which were all identified based on data available in the Maize Genomics Sequencing Project (MGSP) database. Cell wall carbohydrate genes were identified based on their Arabidopsis orthologs and previous research in C3 grasses by MITCHELL *et al.* (2007). Transcription and regulation factors of cell wall genes were similarly identified based their orthologs described both in Arabidopsis and woody species. All these genes were mapped in silico considering their physical position in the MGSP database. Physical positions of previously described QTL for cell wall degradability, lignin and *p*-hydroxycinnamic acid contents were also searched for based on the position of the flanking marker in the MGSP database and distances between QTL and flanking markers. While only a few lignin pathway genes mapped to QTL positions, several colocalizations were shown between QTL and transcription factor physical positions. This last result is in agreement with expression studies which highlighted that several genes in the lignin pathway are simultaneously under-expressed in lines with higher cell wall degradability, likely corroborating an upstream regulation rather than co-regulation phenomena. However, none of these possible candidate genes have yet been validated and many QTL still do not have relevant candidates. A lot of relevant candidate genes are still to be discovered among those involved in lignin pathway gene regulation, in regulation of lignified tissue assembly, and in cell wall carbohydrate biosynthesis and deposition. In addition, in spite of their critical involvement in maize cell wall assembly and degradability, genes involved in ferulic acid biosynthesis and linkages with other cell wall components are little known.

KEYWORDS: Maize; Cell wall; Silage; Biofuel; Bioethanol; Lignin; Monolignol; *p*-coumaric acid; Ferulic acid; Cellulose; Arabinoxylans, Digestibility; Degradability; Genetic resources; Genetic engineering; Plant breeding; Cattle feeding; Intake; Drought tolerance.

INTRODUCTION

Grasses are the basis of energy nutrition of wild and domesticated herbivores, with grazing during spring and early autumn and silage maize feeding during the long periods without meadow growth. Hence, in France, 90% of milk is produced by cows fed on silage maize. Recent economic and environmental factors have also strengthened the need to produce sustainable substitutes to fossil fuels. The most important resource for biofuel in the future will be based on the use of ligno-cellulose products and by-products from grasses and trees. However, although biomass contains almost the same amount of gross energy as do grains per unit of dry matter, the energy value of biomass only ranges from 80% (leafy ray-grass) to 33% (wheat straw) of maize

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grain value in digestive tracts of animals or in industrial enzymatic fermenters. The lower energy value of cell wall parts, in comparison with grains, by micro-organism or industrial enzymes in animal rumen or fermenters, is related to their content in phenolic compounds which induce limited and variable carbohydrate degradability. Lignins contribute to the structural integrity of tissues and impart hydrophobicity to vascular elements. Lignin associations with other matrix components, together with phenolic linkages between cell wall carbohydrates, greatly influence cell wall properties. Embedding between phenolics and carbohydrates thus prevent physical access of enzymes to cell wall carbohydrates and strongly limit their enzymatic hydrolysis. In addition, lignins adsorb hydrolytic enzymes on their surfaces, and consequently have a second negative effect on carbohydrate degradation. Moreover, lignin degradation products resulting from industrial pretreatment inhibit ethanologenic fermentations (Keating *et al*., 2006; Li *et al*., 2008).

"Corn" (or maize, *Zea mays* L.) is likely the plant species in which genetic improvements for agronomic traits were the most remarkable during the last century in the USA (Russell, 1984; Troyer, 1999; Troyer and Rocheford, 2002) and over the last five decades in Europe (Barrière *et al*., 1987, 2005, 2006; Derieux *et al*., 1987a,b). In forage maize (Barrière *et al*., 1987, 2004a, 2005), the genetic progress in yield was close to 0.17 t/ha per year for hybrids registered in France between 1986 and 2000 (1986 is the first year with registration tests including whole plant traits). Before 1986, forage yield improvement was correlative to the genetic progress in grain and was nearly equal to 0.10 t/ha per year (Barrière *et al*., 1987). Feeding value was not considered for forage maize registration until 1998 in France, and a significant drift towards lower cell wall digestibility values was observed between 1975 and 2000 (Barrière *et al*., 1997, 2004a). In the USA, a forage yield increase of nearly 0.14 t/ha per year since 1930 has been shown (Lauer *et al*., 2001). Conversely to what was observed in Europe, no change in the cell wall digestibility of plants was observed, despite the fact that major improvements in stalk standability and stalk rot resistance were achieved during the same period. This discrepancy between European and US results is likely due to differences in genetic backgrounds of maize hybrids and different evolutions of hybrid germplasm in Europe and in the USA.

Maize cropping for silage use in Europe is mostly based on varieties bred for whole plant agronomic and quality traits, most of which have average cell wall degradability. Besides woody plants, the future biofuel production will be based on the use of straw available after grain harvest of maize, wheat, rice, other cereal crops, and possibly sunflower or rapeseed. It will also be based on high yielding late maize, sorghum, sugarcane, switchgrass, and other C4 grasses. In any case, maize genetic improvement allowing increased animal or biofuel productions based on the non-grain part of plants requires breaking down the cell wall degradability trait into its underlying biochemical and genetic components. Breeding maize with more degradable cell walls will be more efficient when the cell wall building rules are clear, with a better understanding of the genetic and molecular mechanisms involved in each maize compound biosynthesis and cross-linkage. Gene discovery is thus a major objective for efficient silage and biofuel breeding programs including marker assisted selection (MAS), SNP (Single nucleotide polymorphism) investigations, genetic resources management, and genetic engineering. Maize cell wall gene search was investigating through in silico mapping based on the Maize Genome Sequencing Project (MGSP) database (www.maizesequence.org, release 3b.50). Identification of maize clones (ACnumber) containing sequences of maize gene or rice orthologs was done with the "blast" tool of PlantGDB (www.plantgdb.org). Searches of maize orthologous genes of dicotyledonous species was investigated with the "tblastn" tool of NCBI (Zea taxid:4575 organism). Positions of SSR markers were searched in the MaizeGDB database (www.maizegdb.org).

PHENOLIC CONSTITUENTS OF MAIZE AND GRASS CELL WALLS

The lignified grass cell wall is a composite material with phenolics, cellulose microfibrils, an amorphous matrix consisting predominantly of hemicelluloses (mainly glucurono-arabinoxylans), and very few pectins. Phenolics are comprised of lignins and cell wall-linked *p*-coumaric (*p*CA) and ferulic (FA) acid derivatives, along with the array of FA dehydrodimer (diFA) derivatives.

Grass lignins are comprised of guaiacyl (G) units derived from coniferyl alcohol, syringyl (S) units derived from sinapyl alcohol, together with lower levels of *p*-hydroxyphenyl units (H) derived from *p*-coumaryl alcohol. 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. The average relative frequencies of each H, G, and S monomeric unit released by thioacidolysis of native lignins of mature maize internodes were shown nearly equal to 2.5, 37.5, and 60.0%, respectively (LAPIERRE, 1993). The low, but appreciable amount of H units, which is nearly five times higher than in dicotyledonous plants, may significantly impact the properties of grass cell walls as these units could increase the frequency of resistant inter-unit bonds. Grass lignins are also characterized by a high content of 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 might be distributed in the cell wall as small and alkali-leachable domains. Despite the fact that they are often referred to as branched three-dimensional polymers, lignins are in fact largely linear. The two only known branching structures in lignins are the 5-5 and 4-O-5 bonding patterns, which cannot be formed without the participation of at least one G (or H) unit (RALPH *et al.*, 2008a). Between these branching points, there are linear lignin fragments essentially made of H, G or S units essentially linked by β-1, β-5, β-β and β-O-4 linkages. In addition, the incorporation of free FA in lignins through bis-8-O-4 cross-coupling provides a third branching point, which occurs at low levels in normal plants, but may build up in CCR-deficient angiosperms (RALPH *et al.*, 2008b).

Together with the importance of H units, the participation of *p*-hydroxycinnamates in cell wall composition and organization of the lignified tissues is certainly the most specific trait of grass lignification. Among cell wall-linked *p*-hydroxycinnamates, *p*-coumarate is mainly esterified to the γ-position of the phenylpropane side chain of S lignin units. Most *p*-coumarate accretion occurs in tandem with lignification and *p*-coumarate accumulation is thus a relevant indicator of lignin deposition. In maize, 25 to 50% of S lignin units may be acylated by *p*CA. This acylation occurs at the monolignol level (LU and RALPH, 1999; MORREEL *et al.*, 2004; GRABBER and LU, 2007; MARTINEZ *et al.*, 2008) and has a marked influence on the bonding mode of S lignin units, on the spatial organization of lignins and on their capacity to interact with polysaccharides. In non-grass cell walls, sinapyl alcohol has a pronounced tendency to be involved in β-O-4 end-wise type coupling upon peroxidasic polymerization. This tendency does not seem to apply to grass cell walls which have a lower content in β-O-4 bonds than the woods of dicotyledonous angiosperms in spite of similar S levels. The *p*-coumaroylation of sinapyl alcohol at C does not seem to significantly interfere with its coupling reactions at the β-position, but post-coupling reactions may be significantly altered (RALPH *et al.*, 2008a).

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 (MACADAM 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. Lignins and arabinoxylans are secondarily bridged through FA ether-linkages at the β-position of G units. Ferulate and diferulates thus provide points of growth for the lignin polymer and direct cell wall cross-linking (RALPH *et al.*, 1992, 1995; JACQUET *et al.*, 1995). 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. Moreover, the presence of ferulates linked to arabinosyl side chains of arabinoxylans provides a convenient and reliable way of cross-linking these polysaccharide chains. Over 50% of wall ferulates can undergo dehydrodimerization and arabinoxylans are thus extensively cross-linked by ferulate dimerization in mature cell walls (GRABBER *et al.*, 2004).

GENETIC VARIATION FOR PHENOLIC TRAITS AND CELL WALL DEGRADABILITY IN MAIZE PLANTS

Large variations in phenolic contents have been shown between maize inbred lines and hybrids (DHILLON *et al.*, 1990; GROTEWOLD and PETERSON, 1994; LUNDVALL *et al.*, 1994; MÉCHIN *et al.*, 2000; FONTAINE *et al.*, 2003a,b; FREY *et al.*, 2004; RIBOULET *et al.*, 2008; BARRIÈRE *et al.*, 2009). Among lines, a variation higher than 40% of average values was observed for ADL/NDF lignin content (Table 1), rang-

TABLE 1 - *Genetic variation for cell wall degradability (IVNDFD) and phenolic traits in a set of early and medium early maize lines, harvested at silage maturity after ear removing and illustrating extreme values for cell wall related traits (unpublished data of INRA Lusignan, field experiments in 2006 and 2008, All trait F genotype are significant at P < 0.001; Trait highest and lowest values are underlined; ADL/NDF = acid detergent lignin / neutral detergent fiber, KlaL/NDF Klason lignin / Ndf; pCa = p-coumaric acid, esterFA and etherFA = esterified and etherified ferulic acids,8-O-4 diFA = 8-O-4 diferulic acid, pHb = p-hydroxybenzaldehyde, Va = vanillin, Sg = syringaldehyde, all traits expressed as mg/g NDF; M13 Dent = Minnesota13 Dent, N Flint = Northern flint, Arg Flint = Argentina Flint).*

	Pedigree	IV NDFD	ADL/ NDF	KlaL/ NDF	*p*CA	Ester FA	Ether FA	8-O-4 diFA	*p*Hb	Va	Sg
F7084	M13 Dent x Flint	39.23	4.29	13.02	10.24	6.05	1.36	0.31	1.16	4.83	5.73
F4	Northern Flint	39.23	4.46	12.50	8.24	4.93	1.21	0.24	1.30	5.45	6.33
F7086	Flint x N Flint	38.98	4.70	14.20	11.10	5.94	1.21	0.28	1.31	5.88	6.32
F7087	Flint x N Flint	38.85	3.83	13.85	12.36	6.68	1.62	0.35	1.23	5.47	7.09
F324	Flint x Arg Flint	38.08	5.21	16.02	10.61	5.65	1.13	0.25	1.47	5.80	6.41
F7082	M13 Dent x BSSS	37.61	3.66	12.63	11.81	6.88	1.12	.25	1.55	5.97	6.80
F7085	M13 Dent x Flint	35.71	4.50	13.55	12.40	6.18	1.33	0.28	1.47	6.10	7.27
F7066	Flint x BSSS	35.70	4.61	14.54	7.65	6.14	1.24	0.26	1.22	5.86	5.30
F66	Sost Flint	34.84	4.81	14.50	8.07	5.11	1.42	0.24	0.99	5.76	6.15
F7019	M13 Dent x BSSS	34.33	4.86	13.13	12.36	6.59	1.41	0.25	1.62	7.33	7.56
W117	M13 Dent	34.27	4.80	14.48	12.61	6.24	1.32	0.25	1.67	5.98	7.08
F7081	M13 Dent x BSSS	33.81	4.37	12.07	13.76	6.99	1.28	0.24	1.75	6.98	7.90
F7058	M13 Dent	33.62	4.40	13.89	14.43	6.87	1.28	0.26	1.86	6.61	8.15
F286	Flint x Arg Flint	33.39	5.07	14.87	11.35	5.86	1.35	0.25	1.67	6.89	7.24
F113	M13 Dent	32.71	4.87	13.88	12.01	6.16	1.34	0.25	1.60	7.27	7.88
F7025	Iodent x M13 Dent	32.60	5.58	15.19	14.00	6.34	1.34	0.24	1.90	7.11	8.93
F7012	Complex Flint	31.47	6.04	18.26	9.00	4.82	1.41	0.24	1.25	5.68	6.04
F2	Lacaune Flint	31.28	5.07	14.99	11.56	5.78	1.47	0.24	1.58	7.29	8.02
F838	Complex Dent	30.38	5.45	15.17	15.71	6.49	1.50	0.20	2.13	8.17	9.07
F271	Canadian dent	28.70	5.04	14.59	11.00	5.92	1.38	0.23	1.69	6.63	8.16
F7037	Complex Dent	27.56	5.12	13.96	14.73	6.26	1.43	0.23	1.82	7.97	9.97
EA1301	Portuguese Flint	25.24	7.09	15.53	12.76	4.11	2.46	0.15	2.09	8.12	11.10
Fl222	Private Flint	25.22	5.84	15.60	13.14	5.89	1.45	0.20	1.86	8.09	9.50
F268	Complex Flint	25.08	6.38	17.14	10.62	4.76	1.67	0.22	1.47	6.90	8.61
F7033	Complex Dent	23.47	6.12	16.12	13.82	6.02	1.73	0.24	2.11	9.10	9.78
F874	Complex Dent	22.96	6.01	16.12	15.72	6.31	1.22	0.23	2.15	7.72	10.86
Confidence limit		1.36	0.34	0.90	0.71	0.27	0.10	0.02	0.20	0.40	0.72

ing from nearly 4% in lines F4 and F7084 to nearly 7% in the line EA1301 [NDF is neutral detergent fiber and ADL is acid detergent lignin according to GOERING and VAN SOEST (1970)].

Partly in correlation with lignin content variation, a 2-fold variation was observed for pCA content. Variation in esterified and etherified FA (esterFA and etherFA) contents illustrated both a possible variable biosynthesis of FA linked to arabinoxylans (FAXX) and, at a given availability of FAXX, a greater proclivity to crosslink with G unit of lignins. Variation for the recovery yield of each H, G or S lignin-derived monomer after nitrobenzene oxidation was similarly of a 2-fold range between maize lines. Heritability of phenolic contents is high in maize stover, as illustrated by data obtained in the RIL F838 x F286 progeny (BARRIÈRE *et al*., 2008) with values ranging from 0.81 (H unit content) to 0.91 (*p*CA content).

As observed for phenolic contents, large genetic variations in the *in vivo* or *vitro* cell wall digestibility of maize plants have been shown (LUNDVALL *et al*., 1994; BARRIÈRE *et al*., 1997, 2004a; ARGILLIER *et al*., 2000; RIBOULET *et al*., 2008), with similarly, small genotype x environment interaction effects com-

pared to main effects. From a long term experiment based on 478 hybrids, the *in vivo* cell wall digestibility in maize (estimated as NDF digestibility, or NDFD) nearly doubled from 32.1 to 60.4% with an average value equal to 48.8% (Barrière *et al*., 2004a). The *in vitro* cell wall digestibility (IVNDFD) also nearly doubled from 22.1 to 39.2% with an average value equal to 32.5% (Table 1) in a set of 26 lines representing probably the largest known variation for this trait [IVNDFD is estimated according to Struik (1983) as (100 x (ES - (100 - NDF) /NDF), based on the enzymatic solubility (ES) of Aufrère and Michalet-Doreau (1983)]. Heritability of *in vivo* or *in vitro* cell wall digestibility traits ranged from 0.65 to 0.90, and it was at least equal to that of yield (Roussel *et al*., 2002; Barrière *et al*., 2008).

Besides cell wall degradability, voluntary intake is a primary nutritional factor controlling animal production (Minson and Wilson, 1994), which is also affected by the phenolic composition of the cell wall. The regulation of intake in cattle is controlled first by the time the silage needs to be broken down in the mouth and swallowed, and secondly the time this forage is retained in the rumen and ruminated until particles reach a size close to 1 mm and escape out of the rumen through the digestive tract (Minson and Wilson, 1994; Jung and Allen, 1995; Fernandez *et al*., 2004). Any traits that make plant fiber particles physically strong and difficult to reduce in size are therefore involved in variation of intake. Variations in cell wall digestibility thus explain nearly one half of intake variations in cows (Barrière *et al*., 2003). In addition, scattered but convergent results support the hypothesis that the second half of genetic variations for intake is explained by plant tissue friability and susceptibility to crushing. These specific characteristics are likely to be present at a high level in rare hybrids (such as DK265 and probably Bahia) and explain their exceptional intake (Ciba-Semences, 1990, 1995; Barrière *et al*., 1995, 2004b).

Genetic variation for cell wall degradability has been related to genetic variation for phenolic traits. Lignin content is the first trait related to cell wall degradability, but breeding for an overly much reduced lignin content has negative consequences on other agronomic traits. However, variations in lignin content are not sufficient to explain variations in cell wall degradability. The correlation between cell wall degradability and *p*CA content is negative and often higher than the correlation with lignin content (Fontaine *et al*., 2003b; Riboulet *et al*., 2008). In addition to being a probable direct effect of S unit acylation on lignin structure, *p*CA content is likely a relevant indicator of secondary tissue lignification. Correlation between cell wall degradability and ether FA in maize is also negative, and the role of ferulate cross-linking was "tentatively estimated to account for nearly one half of the inhibitory effects of lignin on cell wall fermentation" (Grabber *et al*., 2009). Breeding for a reduced level of ether-linked ferulate is thus expected to improve maize cell wall degradability as it has been observed in bromegrass (Casler and Jung, 1999). In addition, ferulate cross-linkages were considered to be involved in stalk stiffness (Grabber *et al*., 1995; Grabber *et al*., 2000; MacAdam and Grabber, 2002) and consequently it could impede plant friability and silage maize intake. Attempts to understand the impact of lignin structure, commonly described by ratios between H, G, S units, on the susceptibility of the cell wall to enzymatic hydrolysis has led to conflicting results and been considered of little importance (Méchin *et al*., 2000; Grabber *et al*., 1997, 2009). However, an increased proportion of H units likely contributes to the lowering of cell wall degradability (Riboulet *et al*., 2008). Similarly, based on correlation data, an increase in S units appears to have a similar negative impact (Riboulet *et al*., 2008). A higher proportion of S units in lignins might thus indicate a higher proportion of mature secondary wall in tissues.

GENES INVOLVED IN MAIZE CELL WALL COMPONENT BIOSYNTHESIS

Genes involved in monolignol and lignin biosynthesis

Monolignol biosynthesis is firstly dependent on the availability of substrates upstream in the phenylpropanoid pathway. The shikimate pathway links the carbohydrate metabolism to the biosynthesis of aromatic amino acids (phenylalanine, tyrosine, and tryptophan) and, consequently, to the phenylpropanoid pathway. The carbon flow in the shikimate pathway, the regulations of the shikimate pathway genes, and the phenylalanine supply to phenylalanine ammonia lyase (PAL) enzymes may thus directly affect phenylpropanoid biosynthesis. Successive steps including hydroxylation and methylation on the aromatic ring lead to the production of three monolignols which are polymerized into lignins (Fig. 1).

Most of the genes involved in monolignol

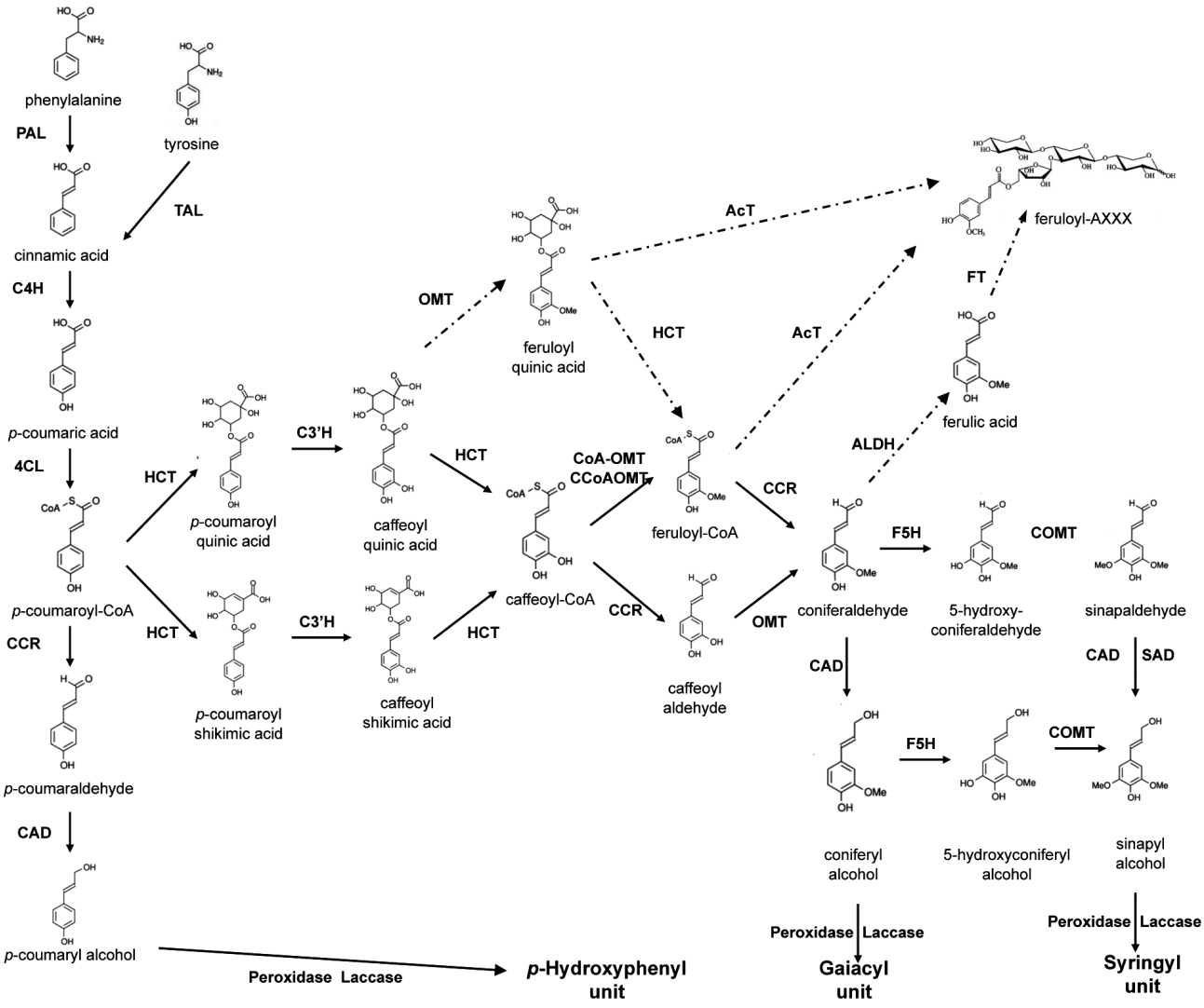

FIGURE 1 - Pathways of monolignols and *p*-hydroxycinnamic acid biosynthesis in maize. Routes towards coniferyl and sinapyl alcohol via feruloyl-CoA (catalyzed by CCoAOMT), and route towards *p*-coumaric alcohol are considered to be established. Another route towards sinapyl alcohol biosynthesis via caffeoyl aldehyde (catalyzed by an unknown OMT) is hypothesized, in agreement with several literature data. Different hypothetical routes from caffeoyl quinic acid or caffeoyl-CoA towards ferulic acid biosynthesis are also proposed, based on available maize and dicotyledonous plant data.
PAL = phenylalanine ammonia lyase, TAL = tyrosine ammonia lyase, C4H = cinnamate 4-hydroxylase, 4CL = coumaroyl-CoA by 4-CoA ligase, HCT = hydroxycinnamoyl-CoA shikimate/quinate hydroxycinnamoyl transferase, C3'H = *p*-coumaroyl-shikimate/quinate 3'-hydroxylase, CCoAOMT = caffeoyl-CoA O-methyltransferase, CoA-OMT = CoA O-methyltransferase, CCR = cinnamoyl-CoA reductase, F5H = ferulate 5-hydroxylase, COMT = caffeic acid O-methyltransferase, CAD = cinnamyl alcohol dehydrogenase, AcT = acyltransferase, ALDH = aldehyde dehydrogenase, FT = feruloyl transferase, AXXX = arabinoxylan chain.

biosynthesis belong to small multigene families. After the shikimate pathway, the first step of monolignol biosynthesis occurs with the deamination of L-phenylalanine into cinnamic acid by a PAL enzyme. Maize PAL also has a tyrosine ammonia lyase (TAL) activity (HIGUCHI *et al*., 1967; ROESLER *et al*., 1997), catalyzing tyrosine deamination into *p*CA. Six PAL genes, which correspond to three groups of probably duplicated genes, are present in the maize genome and expressed in maize lignifying tissues (Table 2). A one-bp deletion in the second exon of the ZmPAL gene, introducing a premature stop codon, has been associated with higher plant digestibility (ANDERSEN *et al*., 2007). The hydroxylation of cinnamic acid is catalyzed by a cinnamate 4-hydroxylase (C4H, two genes) and yields *pCA*, which

TABLE 2 - *Survey of genes involved in monolignol and common phenylpropanoid compound biosynthesis based on MaizeWall and MGSP (Maize Genome Sequencing Project) databases.*

Gene	bin	MGSP contig	Contig position	MGSP clone and gene
Phenylalanine amonia lyase				
PAL2a (pal2 locus)	2.03	ctg74	22.2 Mb	AC213314.3_FG039
PAL2b (pal2 locus)	2.03	ctg74	22.2 Mb	AC213314.3_FG037
PAL3a (pal3 locus, ZmPAL)	4.05	ctg177	132.4 Mb	AC195904.2_FG028
PAL3b (pal3 locus)	4.05	ctg177	132.4 Mb	AC195904.2_FG030
PAL3c (pal3 locus)	4.06	ctg179	137.5 Mb	AC214752.3_FG046
PAL3d (pal3 locus)	4.06	ctg179	137.5 Mb	AC214752.3_FG047
4-CoA ligase				
4CL1	5.04	ctg225	92.2 Mb	AC209245.3_FG033
4CL2	1.07	ctg44	197.7 Mb	AC191088.3_FG038
4CL3	1.01	ctg5	11.5 Mb	AC190650.3_FG027
4CL4	8.06	ctg362	155.1 Mb	AC225704.2_FG030
4CL5	9.04	ctg380	76.6 Mb	AC212872.3_FG033
Cinnamate 4-hydroxylase				
C4H1	8.03	ctg338	65.2 Mb	AC195798.3_FG039
C4H2	8.08	ctg364	161.4 Mb	AC208713.3_FG021
Hydroxycinnamoyl-CoA transferase				
HCT1	5.05	ctg243	181.4 Mb	AC185460.3_FG034
HCT2	2.04	ctg76	30.5 Mb	AC200505.3_FG032
Coumaroyl shikimate/quinate 3'-hydroxylase				
C3'H1	3.06	ctg138	178.8 Mb	AC200558.3_FG040
C3'H2	6.06	ctg287	148.7 Mb	AC204511.4_FG044
Caffeoyl-CoA O-methyltransferase				
CCoAOMT1	6.01	ctg263	24.0 Mb	AC207735.2_FG031
CCoAOMT2	9.02	ctg373	21.9 Mb	AC205992.3_FG043
CCoAOMT3	2.07	ctg98	180.1 Mb	AC191259.2_FG046
CCoAOMT4	4.08	ctg188	198.8 Mb	AC205996.3_FG037
CCoAOMT5	4.08	ctg188	198.8 Mb	AC205996.3_FG038

is then converted into coumaroyl-CoA by a 4-CoA ligase (4CL, four genes). The down-regulation of the Arabidopsis 4CL1 gene reduced the G unit content in lignins, but did not affect the S unit content (LEE *et al.*, 1997), strengthening the existence of metabolons based on different members of multigene families. Coumaroyl-CoA is converted into caffeoyl-CoA through the formation of quinate or shikimate esters by a hydroxycinnamoyl-CoA shikimate/quinate hydroxycinnamoyl transferase (HCT, two genes). The hydroxylation of these esters to caffeoyl analogues is catalyzed by a *p*-coumaroyl-shikimate/quinate 3'-hydroxylase (C3'H, two genes), giving trans-(*p*-caffeoyl)-shikimic and chlorogenic acids, respectively. The formation of caffeoyl-CoA is obtained by the reverse-active HCT (SCHÖCH *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, has not been established in maize. However, in Arabidopsis, the shikimate ester was converted four times faster than the quinate ester (SCHÖCH *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). Based on dicotyle-

TABLE 3 - *Survey of genes involved or putatively involved in the final steps of monolignols and methyl precursor biosynthesis based on MaizeWall, MGSP (Maize Genome Sequencing Project) databases and* Ranocha et al. *(2001) for HMT genes.*

Gene	bin	MGSP contig	Contig position	MGSP clone and gene
Ferulate 5-hydroxylase				
F5H1	1.07	ctg46	211.8 Mb	AC210173.3_FG032
F5H2	5.03	ctg212	21.5 Mb	AC218041.3_FG037
Caffeic acid O-methyltransferase				
COMT	4.04	ctg164	32.7 Mb	AC203909.2_FG034
Cinnamoyl-CoA reductase				
CCR1 (ZmCCR1)	1.07	ctg44	199.3 Mb	AC192360.3_FG037
CCR2 (ZmCCR2)	7.02	ctg301	41.2 Mb	AC199442.3_FG037
CCR3	4.09	ctg197	231.9 Mb	AC205161.3_FG039
CCR4a	9.03	ctg376	59.3 Mb	AC212136.3_FG032
CCR4b	9.03	ctg376	59.3 Mb	AC212136.3_FG035
CCR5	5.00/01	ctg204	2.9 Mb	AC197125.2_FG036
CCR6	10.03	ctg399	41.3 Mb	AC204873.3_FG038
CCR7	7.03	ctg318	110.4 Mb	AC216049.3_FG047
Cinnamyl alcohol dehydrogenase				
ZmCAD2 (EgCAD2 type)	5.04	ctg229	103.7 Mb	AC230031.1_FG034
ZmCAD1 (EgCAD1 type)	5.04	ctg234	129.2 Mb	AC194671.1_FG025
CAD3 (EgCAD2 type)	9.01	ctg371	17.0 Mb	AC201756.4_FG026
CAD4 (EgCAD2 type)	10.03	ctg400	48.2 Mb	AC208560.2_FG026
CAD5/SAD (EgCAD2 type)	7.02	ctg310	86.0 Mb	AC197840.3_FG030
CAD6/SAD (EgCAD2 type)	2.02	ctg70	9.9 Mb	AC215994.3_FG038
S-adenosylmethionine synthetase				
SAMS	3.04	ctg119	57.1 Mb	AC199526.4_FG030
SAMS	8.03	ctg334	44.7 Mb	AC215179.3_FG023
SAMS	8.05	ctg354	121.2 Mb	AC202164.3_FG034
SAMS	10.03	ctg406	70.1 Mb	AC214838.3_FG041
Homocysteine S-methyltransferase				
HMT1	9.—	ctg—	- Mb	AC233893.1
HMT2	1.05	ctg36	160.5 Mb	AC214142.3_FG034
HMT3	3.04	ctg123	105.1 Mb	AC217431.2_FG014
HMT4	3.06	ctg139	183.4 Mb	AC195676.3_FG039

donous data, shikimate derivatives are therefore probably the preferential substrates in the biosynthesis of maize lignin G and S units.

Long displayed as a metabolic grid (Dixon *et al.*, 2001), the lignin pathway has more recently been based on a caffeoyl-CoA O-methyltransferase (CCoAOMT) hub (Fig. 1). Partially separate pathways leading to G and S units could also be considered, corresponding to different spatio-temporal metabolons (Guo *et al.*, 2001; Parvathi *et al.*, 2001). G unit biosynthesis could be preferentially based on a caffeoyl-CoA methoxylation catalyzed by CCoAOMT activities, while S unit biosynthesis could be preferentially based on caffeoyl-aldehyde methoxylation catalyzed by O-methyltransferase (OMT) activities (Fig. 1, Table 3). OMT involved in this last reaction was first considered as caffeic acid O-methyltransferase (COMT), based on experiments in alfalfa and Arabidopsis (Lee *et al.*, 1997; Guo *et al.*, 2001; Parvathi *et al.*, 2001; Chen *et al.*, 2006; Do *et al.*, 2007). However, ZRP4-like OMT could also be considered to be involved in this step (Barrière

et al., 2007). At least nine ZRP4-like OMT are expressed in maize lignifying tissue. Their role is likely not limited to methylation of suberin sub-unit precursors in plant roots as initially described by HELD *et al*. (1993). Five CCoAOMT genes exist in the maize genome, but CCoAOMT4, which is in duplicate position with CCoAOMT5, appeared to be of little importance in constitutive lignification (CIVARDI *et al*., 1999; GUILLAUMIE *et al*., 2007a,b; RIBOULET *et al*., 2009).

Reductions of *p*-coumaroyl-, caffeoyl-, and feruloyl-CoA thioesters into their corresponding aldehydes are catalyzed by cinnamoyl-CoA reductases (CCR). Among the different CCR genes found expressed in maize lignifying tissue, ZmCCR1 (PICHON *et al*., 1998) was the gene which was the most expressed and the most frequently expressed across stages (GUILLAUMIE *et al*., 2007b; RIBOULET *et al*., 2009). In addition, the CCR3 gene was significantly expressed in young plants and older maturing stalks. Other members of the CCR family possibly correspond to genes mainly involved in defense processes.

In sinapyl alcohol biosynthesis, a ferulate 5-hydroxylase (F5H) catalyzes the 5-hydroxylation of coniferaldehyde (and probably coniferyl alcohol) into 5-hydroxyconiferaldehyde (5-hydroxyconiferyl alcohol, respectively). F5H1 had a strong expression in maize stalks (RIBOULET *et al*., 2009), while F5H2 was mostly expressed in roots (GUILLAUMIE *et al*., 2007b). 5-Hydroxyconiferaldehyde (and 5-hydroxyconiferyl alcohol) is methylated into sinapaldehyde by the COMT. The maize COMT is the only gene of the monolignol pathway which does not belong to a small multigene family. This singularity could corroborate the fact that this gene would be only involved in this step of the pathway, and in a unique metabolon. In fact, the COMT enzyme has no *in vivo* activity on caffeic acid, but is only active on 5-hydroxy-coniferaldehyde in the second methylation step (DAVIN *et al*., 2008). In addition, CCoAOMT enzymes have a strict affinity for CoA-esters (MARTZ *et al*., 1998; MENG and CAMPBELL, 1998; PARVATHI *et al*., 2001). In comparison with COMT, CCoAOMT genes appeared earlier during plant evolution, in agreement with the most primitive appearance of G units comparatively to S units. Only a CoA-OMT has been found in the genome of a cyanobacterium (MENG and CAMPBELL, 1998), and a CCoAOMT gene existed in gymnosperm (LI *et al*., 1999), with an encoded protein having a high catalytic efficiency for caffeoyl-CoA. A COMT related enzyme (AEOMT), having a similar activity on acids and CoA-esters, has also been found in gymnosperms (LI *et al*., 1997). The two closest maize orthologs of the pine AEOMT gene are two genes encoding proteins of unknown function "DUF803" (AC208555.3_FG038 and AC203002.3_FG033) located in ctg285 and ctg346 of chromosomes 6 and 8, respectively. In bm3 COMT disrupted mutant (VIGNOLS *et al*., 1995), the enzymatic affinities of maize CCoAOMT enzymes cannot explain the significant residual content of S units, which reaches 40% of the normal value (KUC and NELSON, 1964; BARRIÈRE *et al*., 2004c). It could then be assumed that at least one ZRP4-like OMT also has activity on 5-hydroxyconiferaldehyde, as it was observed for AEOMT in pine. In agreement with this hypothesis, expression of two ZRP4-like OMT was increased by nearly two fold in bm3 young and silking plants (GUILLAUMIE *et al*., 2007a, and unpublished data).

Reduction of *p*-hydroxycinnamaldehydes leads to the three *p*-coumaryl, coniferyl, and sinapyl alcohols (BOUDET, 2000, 2003; BOERJAN *et al*., 2003). These steps are catalyzed by cinnamyl alcohol dehydrogenases (CAD) considered as active on the three *p*-hydroxycinnamaldehydes. A specific sinapyl alcohol dehydrogenase (SAD) gene has been described once in aspen (LI *et al*., 2001), but this result has not been found in other plants. Two types of CAD genes were described based on investigations in eucalyptus. 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). EgCAD2-type proteins are zinc-containing long-chain alcohol dehydrogenases active as dimers (JORNVALL *et al*., 1987; HAWKINS and BOUDET, 1994). The maize CAD gene ZmCAD2, orthologous to the EgCAD2 gene, is associated with the bm1 mutation and located in bin 5.04 (PROVAN *et al*., 1997; HALPIN *et al*., 1998). It is not yet established whether the bm1 mutation occurred in the ZmCAD2 gene or in a close gene regulating all maize CAD genes (GUILLAUMIE *et al*., 2007a). Four other EgCAD2-type CAD were shown to be expressed in maize, including the two aspen SAD orthologs (GUILLAUMIE *et al*., 2007b), but their specificity of encoded proteins towards sinapaldehyde has not yet been established. The function of EgCAD1-type enzymes in the constitutive lignin pathway is not completely understood. One such gene was recently proven to be also involved in the synthesis of coniferyl alcohol (DAMIANI *et al*., 2005). An EgCAD1-type CAD activity has been described

TABLE 4 - *Survey of genes involved or putatively involved in monolignol export to the cell wall and polymerization based on MaizeWall, TIGR, MGSP (Maize Genome Sequencing Project) databases, and* MITCHELL et al. *(2007) for genes of the PFAM02458 family.*

Gene	bin	MGSP contig	Contig position	MGSP clone and gene
UDP glucuronosyl / UDP glucosyl transferase				
ZmUGT UGT7E2-like1a	4.01	ctg155	3.2 Mb	AC200309.3_FG018
ZmUGT UGT7E2-like1b	4.01	ctg155	3.2 Mb	AC200309.3_FG019
ZmUGT UGT7E3-like1	4.02	ctg157	7.4 Mb	AC200747.4_FG041
ZmUGT	3.06	ctg134	167.7	Mb AC211669.4_FG037
Coniferin β-glucosidase				
β-Glu45/46/47-like1	10.05	ctg413	114.6 Mb	AC195682.4_FG33
β-Glu45/46/47-like2	10.05	ctg413	114.6 Mb	AC195682.4_FG39
β-Glucosidase	3.04	ctg121	85.4 Mb	AC225369.2_FG032
ATP-binding cassette (ABC) transporters				
ABC transpt1 MaizeWall 3024030.2.1	1.12	ctg67	288.9 Mb	AC207669.3_FG035
ABC transpt2 MaizeWall 3871923.2.1	2.03	ctg74	23.2 Mb	AC202416.3_FG043
ABC transpt3 EucaWood ctg6414	8.00	ctg326	0.5 Mb	AC198928.3_FG037
Glutathione S-transferase				
GST II ZmGST17 (Bronze2 like)	-	-	-	no true position
GST III ZmGST22 (Bronze2 like)	10.04	ctg409	80.6 Mb	AC203384.3_FG034
Peroxidase				
ZmPox2	1.04	ctg14	62.7 Mb	AC208576.3_FG024
ZmPox3	6.05	ctg281	118.0 Mb	AC194248.3_FG041
ZmPox4	5.03	ctg219	49.0 Mb	AC195863.2_FG038
ZmPox5	1.05	ctg28	111.3 Mb	AC211264.3_FG038
ZmPox6	3.05	ctg126	129.6 Mb	AC211202.3_FG022
Laccase				
ZmLac1a	3.06	ctg137	171.5 Mb	AC207620.2_FG036
ZmLac1b	3.06	ctg137	171.5 Mb	AC207620.2_FG038
ZmLac2	4.09	ctg197	233.0 Mb	AC211909.2_FG030
ZmLac3	4.08	ctg184	187.7 Mb	AC202105.4_FG027
ZmLac4	1.03	ctg11	45.5 Mb	AC205003.2_FG029

in maize by KANAZAWA *et al.* (1999) and the ZmCAD1 gene is located in the same chromosome bin as the bm1 gene, but nearly 30 Mb downstream (Table 3).

The three *p*-hydroxycinnamyl alcohols are transported from the cytosol to the apoplast as still unidentified conjugates even if monolignol glucosides are often considered to be transport (and storage) forms of monolignols (LIM *et al.*, 2005; ESCAMILLA-TREVINO *et al.*, 2006). Two uridine-diphosphate-glucosyltransferases (UGT) have been shown in Arabidopsis and are capable of glusosylating coniferyl and sinapyl alcohols (LIM *et al.*, 2005; LANOT *et al.*, 2006). These two genes have three orthologs in maize with a duplication of the UGT72E2 orthologous gene (Table 4). In addition, one UGT gene differentially expressed in normal and bm3 internodes (GUILLAUMIE *et al.*, 2008) could be considered as candidate. 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 gene was first identified from a pine xylem library by DHARMAWARDHANA *et al.* (1995). Arabidopsis β-Glu45 and β-Glu46 β-glucosidases (UGT), which are strongly expressed in lignifying organs, were shown to encode proteins with narrow specificity towards the three monolignol glucosides (Es-

CAMILLA-TREVINO *et al*., 2006). The two Arabidopsis β-Glu45 and β-Glu46 genes (together with β-Glu47) correspond to two maize orthologs genes (Table 4). Similarly, another UGT differentially expressed in normal and bm3 internodes is also a possible candidate. ABC transporters are probably involved in the transport of monolignols across membranes (SANCHEZ-FERNANDEZ *et al*., 2001; SAMUELS *et al*., 2002; EHLTING *et al*., 2005). Out of the two maize mostly expressed ABC transporters (GUILLAUMIE *et al*., 2007b), the first one was over-expressed in maize bm3 plants and under-expression in bm2 plantlets, and could therefore be supposed to be preferentially involved in coniferyl alcohol transport. The third one is expressed in xylem tissue of eucalyptus and also has a rice ortholog (RENGEL *et al*., 2009). Because several ABC transporters have a substrate preference for glutathione conjugates (MCGONIGLE *et al*., 2000; SANCHEZ-FERNANDEZ *et al*., 2001; YAZAKI, 2005), a coupled activity could be assumed between glutathione-S-transferases ZmGST17/GST22 and ABC transporters.

Dehydrogenative polymerization of monolignols is mainly driven by peroxidases and/or laccases to form lignins (BOUDET, 2000; CHRISTENSEN *et al*., 2000; BOERJAN *et al*., 2003). The mode of condensation of monolignols is often considered to occur after free radical coupling (FREUDENBERG, 1959), but has also been considered to occur through an ordered radical coupling driven by dirigent proteins (DAVIN and LEWIS, 2000). How monolignol are polymerized is still open to debates and is a matter of some contraversy (DAVIN *et al*., 2008; RALPH *et al*., 2008a). However, the coupling modes leading to the lignin polymers and their specific structure are markedly influenced by the polysaccharidic matrix in which lignification occurs. This so called "template effect" is supported by experimental data obtained for gymnosperm and dicotyledonous angiosperm woods (LAPIERRE *et al*., 1991; AIMI *et al*., 2005; LAWOKO *et al*., 2005). Such information is not available for grass cell walls, but a similar situation seems likely. In addition, sinapyl alcohol is far more rapidly oxidized in the presence of *p*-coumarate, which is then oxidized by peroxidases and transfers the radical to sinapyl alcohol (BOUDET, 2000; HATFIELD *et al*., 2008). Peroxidases have long been considered as the unique class of oxidases involved in lignin polymerization. However, EST sequencing 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 difficult to assign a specific function to any particular oxidase (BOUDET, 2000). When considering genes which were shown to localize and be expressed in maize vascular and lignifying tissues (DE OBESO *et al*., 2003; GUILLAUMIE *et al*., 2007b; RIBOULET *et al*., 2009), five peroxidases and five laccases, including the duplicated ZmLac1 gene, were currently shown in the maize genome (Table 4). The maize laccase genes were orthologs to the poplar lac3 gene, whose down-regulation in poplar induced an important alteration of xylem fiber cell walls, with an increase in soluble phenolic compounds, especially in xylem parenchyma cells (RANOCHA *et al*., 2002).

Genes involved in the S-adenosyl-L-methionine cycles

The methylation of lignin precursors by SAM-dependent O-methyltransferases (S-adenosyl-L-methionine, SAM or AdoMet) consumes large amounts of methyl groups (VAN DER MIJNSBRUGGE *et al*., 2000). The formation of SAM from methionine and ATP is catalyzed by an S-adenosyl-methionine synthetase (SAMS). SAM-dependent transmethylation reactions release S-adenosyl-homocysteine (SAH or AdoHcy), which is a strong competitive inhibititor of COMT and CCoAOMT enzymes (RAVANEL *et al*., 1998; KOCSIS *et al*., 2003). SAH is thus promptly 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). The SAM pool is also regulated by the S-methylmethionine (SMM) "futile" cycle (RANOCHA *et al*., 2001), with the synthesis of SMM by a methyl transfer from SAM to methionine catalyzed by a S-adenosylmethionine:methionine S-methyltransferase (MMT) and the release of SAH. SMM is reconverted to methionine by transferring a methyl group to homocysteine, in a reaction catalyzed by a homocysteine S-methyltransferase (HMT). This set of inter-dependent methionine-related cycles might therefore significantly impact the efficiency of methylation reactions in the lignin pathway and correlatively the quantity of lignins, the S/G ratio, and the ferulate contents. In lignifying tissues, PAL, CCoAOMT, and COMT expression profiles were thus highly correlated with SAMS and HMT profiles in maize or eucalyptus (VINCENT *et al*., 2003; KIRST *et al*., 2004; GUILLAUMIE *et al*., 2007a). However, it would seem that no data has yet shown that methyl group availability could

TABLE 5 - *Survey of genes involved or putatively ferulic acid biosynthesis based on MaizeWall, MGSP (Maize Genome Sequencing Project) databases, and* Mitchell et al. *(2007) for genes of the PFAM02458 family.*

Gene	bin	MGSP contig	Contig position	MGSP clone and gene
Arabinoxylan CoA-acyl transferase (PF02458)				
Ara-CoA-acylT1	3.02/03	ctg112	7.7 Mb	AC213984.3_FG050
Ara-CoA-acylT2	3.03	ctg112	9.0 Mb	AC204355.2_FG023
Ara-CoA-acylT3	3.09	ctg150	212.7 Mb	AC182835.3_FG033
Ara-CoA-acylT4	6.03/04	ctg275	90.7 Mb	AC200122.4_FG040
Ara-CoA-acylT5	6.05	ctg281	121.5 Mb	AC203836.3_FG033
Ara-CoA-acylT6	8.03	ctg328	18.3 Mb	AC211862.4_FG029
Ara-CoA-acylT7	8.03	ctg340	73.4 Mb	AC214128.4_FG038
Ara-CoA-acylT8a	8.05	ctg358	135.2 Mb	AC209719.3_FG030
Ara-CoA-acylT8b	8.05	ctg358	135.2 Mb	AC209719.3_FG031
Aldehyde dehydrogenase				
ZmALDH RF2C	3.09	ctg150	216.4 Mb	AC191038.4_FG032
ZmALDH RF2D	3.09	ctg150	216.4 Mb	AC191038.4_FG034
ZmALDH3 ALDH22A1 ortholog	7.04/05	ctg325	145.5 Mb	AC212124.4_FG031
ZmALDH4 ex BADH	4.05	ctg174	119.5 Mb	AC193454.3_FG038
ZmALDH5	2.05	ctg89	126.6 Mb	AC177866.2_FG039
ZmALDH6	2.06/07	ctg97	175.2 Mb	AC196479.3_FG042
ZmALDH7	4.08/09	ctg193	218.4 Mb	AC204269.3_FG043
O-methyltransferase ZRP4-like				
OMT ZRP4-like1	4.03	ctg160	18.7 Mb	AC203063.3_FG037
OMT ZRP4-like2a	2.05	ctg89	125.5 Mb	AC190758.2_FG023
OMT ZRP4-like2b	2.05	ctg89	125.9 Mb	AC207244.3_FG043
OMT ZRP4-like2c	2.05	ctg89	116.6 Mb	AC203920.3_FG029
OMT ZRP4-like3a	9.04	ctg383	87.7 Mb	AC210104.3_FG034
OMT ZRP4-like3b	9.04	ctg383	87.7 Mb	AC210104.3_FG035
OMT ZRP4-like4	4.08	ctg188	197.0 Mb	AC208511.3_FG031
OMT ZRP4-like5a	6.06	ctg288	151.7 Mb	AC187896.3_FG033
OMT ZRP4-like5b	6.06	ctg288	151.7 Mb	AC187896.3_FG032
OMT ZRP4-like5c	6.06	ctg288	151.7 Mb	AC187896.3_FG034

be a limiting factor in monolignol biosynthesis. Based on MaizeWall database, four SAMS genes were identified in maize (Table 3). Similarly four HMT (ZmHMT1-4) have been identified in maize (Ranocha *et al*., 2001), but there is still no data available for the ZmHMT1 (AF297044) gene (Table 3).

Genes involved in p-coumarate and ferulate biosynthesis

The biosynthesis of *p*CA from phenylalanine or tyrosine is clearly established, even if different members of each multigene family may be involved in the pathway resulting in *p*-coumaryl alcohol and in the pathway resulting in *p*Ca involved in acylation of sinapyl alcohol. Genes encoding acyltransferases (AcT) involved in acylation at the γ-carbon of monolignol side-chain are still unknown, but a candidate enzyme with higher affinity towards sinapyl alcohol than towards coniferyl alcohol has been found (Martinez *et al*., 2008; Hatfield *et al*., 2009). AcT was shown to use an activated acid (*p*-coumaroyl-CoA) to form the corresponding sinapyl *p*-coumarate (Hatfield *et al*., 2009).

Unlike *p*CA, the pathway allowing FA biosynthesis is still largely unknown. Results obtained with the maize bm3 mutant strongly suggest that FA is not biosynthesized by a COMT-catalyzed methyla-

tion of a caffeic precursor. The disruption of the COMT gene does not affect the FA content of mutant plants (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*., 2000). In alfalfa, the content in FA, which is present in the cell wall in nearly 100 times lower amounts than in maize, is significantly decreased in HCT and C3'H down-regulated plants, but not in CCoAOMT down-regulated plants (Chen *et al*., 2006). This result suggests that ferulate originates either from a route branching before the CCoAOMT step or from a CCoAOMT gene escaping the RNA interference. However, this last hypothesis is not the most probable as no CCoAOMT activity was shown towards caffeoyl-CoA in CCoAOMT down-regulated plants, despite the fact that G unit thioacidolysis yield was only reduced (Chen *et al*., 2006). In addition to variation in lignin content and decrease in 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). The FA biosynthesis pathway therefore appears closer to that of syringyl alcohol than that of coniferyl alcohol. Both ferulic acid and syringyl alcohol pathways might escape the CCoAOMT step.

A complementary hypothesis can be considered from investigations in the Arabidopsis REF1 plants. REF1 mutant, which has a reduced content in soluble sinapate esters, is affected in a sinapaldehyde dehydrogenase and the REF1 protein exhibited *in vitro* both sinapaldehyde and coniferaldehyde dehydrogenase activities (Nair *et al*., 2004). Sinapic and probably ferulic acids in Arabidopsis are thus derived from oxidation of the corresponding aldehydes, but it is not yet known if this ALDH pathway also exists in maize and grasses. In maize, three mitochondrial and two cytosolic ALDH (ALDH2C and 2D) orthologs of the REF1 gene have been described (Skibbe *et al*., 2002; Nair *et al*., 2004). Two other REF1 orthologs which were originally, but falsely, considered to be a betaine aldehyde dehydrogenase (Kirch *et al*., 2004; Kirch *et al*., 2005) were also shown to be expressed in maize (Guillaumie *et al*., 2007b; Riboulet *et al*., 2009). In addition, three ALDH which have a sequence that is more different from the REF1 one might be hypothesized as more specific grass candidate genes (Table 5). The putative involvement of ALDH genes in grass ferulate biosynthesis has still to be considered, but not enough data is available to draw any definite conclusions.

Feruloyl-glucose was considered to be a precursor for the intracellular feruloylation of arabinoxylans based on experiments with radio-labeled FA given to suspension-cultured wheat cells (Obel *et al*., 2003). However, the formation of feruloyl-glucose might occur in these conditions as a storage or detoxification compound when cells are fed FA. From cell cultures fed [^{14}C]cinnamates, the *p*-coumarate to caffeate and ferulate conversion did not occur at the free acid level, but appeared to involve conjugates of the acids which are possibly CoA-esters (Fry *et al*., 2000). [^{14}C]Feruloyl and [^{14}C]diferuloyl arabinoxylan-bound residues were indeed found in cell culture fed [^{14}C]cinnamates. Moreover, a putative feruloyl-CoA-arabinoxylan-trisaccharide *O*-hydroxycinnamoyl transferase activity (Yoshida-Shimokawa *et al*., 2001) has been found in suspension-cultured rice cells fed feruloyl-CoA and arabinoxylan-trisaccharide (AXX) with the formation of feruloyl arabinoxylan-trisaccharide (FAXX). In addition, genes involved in arabinoxylan feruloylation were tentatively identified as acyltransferase (AcT) genes specifically expressed in grasses in contrast to dicotyledons in which this particular function is supposed to be missing (Mitchell *et al*., 2007). One of the most differentially expressed groups of grass genes was a member of the Pfam family PF02458 (Finn *et al*., 2008) encoding CoA-dependent AcT including hydroxycinnamyl transferases (Yang *et al*., 1997). These genes are therefore the strongest candidates for encoding enzymes catalyzing the transfer of a feruloyl group onto an arabinoxylan chain in grass cell walls. Based on candidate genes considered in C3 grasses by Mitchell *et al*. (2007), nine genes were retained in maize (Table 5). These results together with enzymatic approaches strengthen feruloyl-CoA as the feruloyl donor in arabinoxylan feruloylation and consequently feruloyl-CoA as the end compound in ferulate biosynthesis. 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 (Encina and Fry, 2005) and/or laccases/O_2.

Several routes leading to maize and grass ferulate derivatives can thus be hypothesized (Fig. 1). Taking into account investigations in CCoAOMT down-regulated plants and the Arabidopsis REF1 mutant, ferulate biosynthesis would include a CCR-catalyzed conversion of caffeoyl-CoA into caffeoyl-aldehyde, an OMT-methylation of caffeoyl-aldehyde into coniferaldehyde, and lastly the oxidation of

TABLE 6 - *Survey of genes putatively involved in xylose and arabinose biosynthesis based on MaizeWall, TIGR, MGSP (Maize Genome Sequencing Project) database.*

Gene	bin	MGSP contig	Contig position	MGSP clone and gene
UDP-D-glucose dehydrogenase (G6DH)				
G6DH1	1.10	ctg58	267.1 Mb	AC191342.2_FG031
G6DH2	5.01/02	ctg206	7.0 Mb	AC231372.2_FG031
G6DH3	1.08	ctg50	237.8 Mb	AC195917.2_FG040
UDP-D-Glucuronic Acid decarboxylase				
UDP-GlcA-dcb1	9.05	ctg387	106.0 Mb	AC206842.3_FG048
UDP-GlcA-dcb2 AtUXS2 ortholog	8.03	ctg337	62.3 Mb	AC213855.4_FG034
UDP-GlcA-dcb3	3.04	ctg119	59.2 Mb	AC214538.4_FG046
UDP-GlcA-dcb4	9.05	ctg387	106.0 Mb	AC187803.3_FG040
UDP-GlcA-dcb5	3.06	ctg138	177.4 Mb	AC210170.5_FG044
UDP-GlcA-dcb6	1.03	ctg11	40.4 Mb	AC208471.4_FG032
UDP-D-xylose-4-epimerase (MUR4-like)				
MUR4-like1	10.06/07	ctg417	125.8 Mb	AC183941.4_FG038
MUR4-like2	2.02	ctg70	8.8 Mb	AC188831.3_FG041
MUR4-like3	1.03	ctg11	46.4 Mb	AC202090.4_FG043

coniferaldehyde into ferulic acid in an ALDH-catalyzed reaction. However, this pathway conflicts with convergent results showing probable feruloylation of arabinoxylans from feruloyl-CoA while sinapoyl-glucose is the intermediate in the biosynthesis of sinapoyl-malate (STRACK, 1977, quoted in CHAPPLE *et al.*, 1992). A devoted metabolon with a grass specific CoA-OMT involved in the biosynthesis of the feruloyl moiety for arabinoxylan feruloylation might then be hypothesized, including the involvement of ZRP4-like OMT as CoA-OMT enzymes (Table 5). Another speculative pathway might thus be considered, based on an OMT catalyzed methylation of caffeoyl-quinic (or chlorogenic) acid into feruloyl-quinic acid followed by its HCT catalyzed conversion into feruloyl-CoA. Chlorogenic acid has been described in maize and implicated with the C-glycosyl flavone maysin in the corn earworm antibiosis (SZALMA *et al.*, 2005). Unlike feruloyl-quinic acid, the corresponding feruloyl-shikimic derivative was not described in any of the investigated plant species (DING *et al.*, 2001; CAMPA *et al.*, 2003; NIGGEWEG *et al.*, 2004; CLIFFORD *et al.*, 2006, 2007).

Genes involved in cell wall carbohydrate biosynthesis

Putative genes encoding for enzymes catalyzing the early steps of cellulose and xylan synthesis have been identified in different plant species. The nucleotide sugar interconversion pathway comprises a set of enzymatic reactions by which plants synthesize activated monosaccharides as precursor elements of cell wall polysaccharides from photosynthesis and D-fructose-6-P (REITER and VANZIN, 2001; REITER, 2008). UDP-D-glucose (UDP-D-Glc), which is at the basis of cellulose and arabinoxylan biosynthesis, is produced from D-fructose-6-P via D-glucose-6-P and D-glucose-1-P in three successive reactions catalyzed by phosphoglucose isomerases, phosphoglucomutases, and UDP-D-Glc pyrophosphorylases. UDP-D-Glc is also available from sucrose and uridine diphosphate (UDP) in the reversible reaction catalyzed by sucrose synthases. Nucleotide sugars are then the substrates which are used for the elongation of carbohydrate chains by UDP-glycosyltransferases (KAWAKITA *et al.*, 1998; GIBEAUT, 2000).

Hemicellulose polysaccharides are formed from UDP-D-glucose in the Golgi apparatus and are exported to the external surface of the membrane in Golgi vesicles (DENNIS and BLAKELEY, 2000). UDP-D-xylose is thus produced from UDP-D-glucose in a set of two reactions. UDP-D-glucose is converted into UDP-D-glucuronic acid (UDP-D-GlcA) in a reaction catalyzed by UDP-D-glucose dehydrogenases for which three genes were found in the maize

genome. UDP-D-GlcA is next converted into xylose in a reaction catalyzed by UDP-D-GlcA decarboxylase for which five putative genes were found in maize (Table 6). In addition, UDP-D-xylose can be converted into UDP-L-arabinose in a reversible reaction catalyzed by an UDP-D-xylose-4-epimerase. In Arabidopsis, a UDP-D-xylose-4-epimerase gene was shown to be affected in the MUR4 mutant (BURGET *et al*., 2003) for which three orthologous genes were shown in maize. The MUR4-like1 gene is characterized by a large number of EST in the MGSP database.

Maize genes involved in secondary wall arabinoxylan chain biosynthesis are not really known, and putative candidate can be mainly considered based on bio-analysis investigations. The biosynthesis of the β-1,4-xylan backbones is catalyzed by UDP-D-xylose:1,4-β-D-xylan 4-β-D-xylosyltransferase ("xylan synthases", GT43 glycosyltransferase family), using uridine 5'-diphosphoxylose (UDP-Xyl) as the donor substrate (URAHARA *et al*., 2004). In Arabidopsis, the IRX9 and IRX14 mutations result in a deficiency in xylan xylosyltransferase (XylT) activity, thus leading to a defect in the elongation of the xylan backbone (BROWN *et al*., 2007; LEE *et al*., 2007a). Several genes of the glycosyltransferase GT43 family were thus shown to be more specifically expressed in grasses (MITCHELL *et al*., 2007), corresponding to five maize genes which are consequently plausible candidates encoding xylan xylosyltransferases (Table 7). The IRX10 mutants of Arabidopsis have similar characteristics to those of the IRX9 mutant, suggesting that IRX10-like glycosyltransferases (GT47 family) might also play a role in elongation of the xylan backbone (BROWN *et al*., 2009; WU *et al*., 2009).

A second classes of glycosyltransferases involved in the xylan synthesis was assigned from the analysis of the Arabidopsis xylan deficient FRA8/IRX7, PARVUS, IRX8 mutants belonging to GT47, GT8, and GT8 families, respectively (BROWN *et al*., 2007; LEE *et al*., 2007b; PERSSON *et al*., 2007; BROWN *et al*., 2009). The FRA8/IRX7, PARVUS, IRX8 mutants all lack the oligosaccharide sequence β-D-Xyl-(1-4)-β-D-(1-3)-α-L-Rha(1,2)-α-D-GalUA-(1,4)-D-Xyl found at the reduced end of xylan chain. It has been suggested that this oligosaccharide may function as a primer for synthesis, and that its absence would lead to impaired xylan chain initiation (PENA *et al*., 2007). Moreover, all Arabidopsis xylan deficient mutants investigated so far were found retaining MeGlcUA but lacking GlcUA, suggesting a link between xylan synthesis and side branch methylation (ZHONG *et al*., 2005; PENA *et al*., 2007; BROWN *et al*., 2007, 2009; WU *et al*., 2009). Members of the glycosyltransferase GT47 family more specifically expressed in C3 grasses were shown by MITCHELL *et al.* (2007) with six maize orthologs including two duplications in bin 8.06 and 3.05 (Table 7). However, these genes were considered as encoding in grasses enzymes with both xylan α-1,2- or α-1,3-arabinosyl transferase functions, allowing the transfer of an arabinosyl residue onto an X(X) chain. Members of the glycosyltransferase GT8 were also found expressed in maize (Table 7) but no function related to xylan synthesis was assigned. As a matter of fact, so far there is no evidence that the oligosaccharide sequence β-D-Xyl-(1-4)-β-D-(1-3)-α-L-Rha(1,2)-α-D-GalUA-(1,4)-D-Xyl is present in grass xylans. In addition, two homologous Arabidopsis genes (RRA1 and RRA2) belonging to the GT77 family were described as encoding an arabinosyl transferase (EGELUND *et al*., 2007). These close genes have three orthologs in maize which can be considered to be putative candidates with an arabinosyl-transferase activity. In addition, specific grass feruloyl-AX β-1,2-xylosyl transferases of the GT61 family may allow the branching of xylan chain (MITCHELL *et al*., 2007; SADO *et al*., 2009), with four putative candidate genes in maize (Table 7).

Cellulose is comprised of hydrogen-bonded β-1,4-linked glucan chains which are synthesized at the plasma membrane by large cellulose synthase (CesA) complexes, using UDP-D-glucose as a precursor. Maize CesA genes were first search for as orthologous genes of the ten Arabidopsis CesA described in the cell wall genomics database of Purdue University (http://cellwall.genomics.purdue.edu/families/2-1.html, Table 8). However, several other CesA genes exist in the maize genome and whether these genes are essential (or equally essential) for the production of cellulose in the primary or secondary cell wall is still not yet known. Deficiency in cellulose biosynthesis and modified orientation or organization of cellulose microfibrils in the cell wall impacted the mechanical quality of plant leaves or stems. In Arabidopsis, the two irregular xylem mutants IRX1 and IRX3 have a reduced stiffness of mature stems correlatively to a cellulose defect in secondary cell walls (TURNER and SOMERVILLE, 1997). The IRX1 and IRX3 Arabidopsis genes encode the catalytic subunits of the cellulose synthase isoforms CesA8 and CesA7, respectively, with the latter being specifically expressed in xylem tissue

(TAYLOR *et al*., 1999, 2000). Other genes also impact cellulose micro-fibril deposition and organization. The fragile fiber FRA1 Arabidopsis mutant, which has a large reduction in fiber mechanical strength without apparent alteration in cell wall composition, is altered in a kinesin-like protein. The latter likely mediates the activity of cortical microtubules in orienting the cellulose microfibrils during differentiation of xylem cells (ZHONG *et al*., 2002). The fragile fiber FRA2 mutant is altered in a gene encoding a katanin-like protein that regulates fiber cell length and wall thickness. The secondary walls of FRA2 fiber cells lack distinct S1, S2, and S3 layers thus indicating that this katanin was considered to be essential for the formation of distinct layers of cellulose microfibrils during secondary wall thickening (BURK *et al*., 2001; BURK and YE, 2002). FRA1 and FRA2 have both orthologous genes in maize (Table 8), whose involvement in cellulose deposition is still unknown. Rice brittle culm1 and maize brittle stalk2 mutants, which have reduced mechanical strengths, are affected in ortholog COBRA-like proteins encoding putative glycosylphosphatidylinositol-anchored proteins (LI *et al*., 2003; CHING *et al*., 2006; SINDHU *et al*., 2007). These COBRA-like proteins were considered to be involved in a patterning of lignin-cellu-

TABLE 7 - *Survey of genes putatively involved in arabinoxylan chain biosynthesis and reorganization based on MaizeWall, TIGR, MGSP (Maize Genome Sequencing Project) databases and* MITCHELL et al. *(2007).*

Gene	bin	MGSP contig	Contig position	MGSP clone and gene
β-1,4-xylan synthase or xylosyltransferase (GT43)				
XylT1 IRX14-like	5.03	ctg219	51.6 Mb	AC197847.3_FG041
XylT2	6.02	ctg271	76.6 Mb	AC207260.3_FG046
XylT3	3.08	ctg145	200.2 Mb	AC191590.3_FG032
XylT4 IRX9-like	8.05	ctg354	123.2 Mb	AC196179.3_FG040
XylT5 IRX9-like	1.03	ctg11	42.9 Mb	AC204421.3_FG027
Xylan α-arabinosyl transferase (GT47, Exostosin-like)				
Xyl-araT2a	8.06	ctg361	151.9 Mb	AC210725.4_FG039
Xyl-araT2b	8.06	ctg361	151.9 Mb	AC210725.4_FG047
Xyl-araT3a IRX10-like	3.05	ctg131	148.5 Mb	AC208612.3_FG032
Xyl-araT3b	3.05	ctg131	148.5 Mb	AC208612.3_FG034
Xyl-araT3c	3.05	ctg131	148.5 Mb	AC208612.3_FG037
Xyl-araT4	2.04	ctg80	61.5 Mb	AC210078.3_FG026
Xyl-araT5 AtARAD1-like	7.04	ctg323	134.9 Mb	AC212234.3_FG036
Transferase FRA8-like	9.07	ctg391	121.3 Mb	AC190637.3_FG031
Xylosyltransferase (GT8)				
Xyl-T1 PARVUS-like	1.09	ctg55	248.2 Mb	AC203404.3_FG036
Xyl-T2 PARVUS-like	1.03	ctg11	46.4 Mb	AC202090.4_FG041
Xyk-T3 IRX8-like	3.04/05	ctg123	109.1 Mb	AC190539.2_FG041
Arabinosyl transferase (GT77)				
AtARR2-like1	1.04	ctg19	73.3 Mb	AC199081.4_FG040
AtARR2-like2	7.04	ctg323	138.3 Mb	AC192356.3_FG041
AtARR2-like3	2.08	ctg104	198.8 Mb	AC231376.1_FG030
Feruloyl-AX -1,2-xylosyl transferase (GT61)				
Feruloyl-XylT1	9.03	ctg375	42.8 Mb	AC189775.3_FG041
Feruloyl-XylT2a	5.03	ctg220	59.9 Mb	AC204720.3_FG028
Feruloyl-XylT2b	5.03	ctg224	80.4 Mb	AC193363.3_FG036
Feruloyl-XylT3	4.09	ctg196	228.0 Mb	AC208418.3_FG038
Feruloyl-XylT4	9.03	ctg375	40.4 Mb	AC215796.3_FG045

TABLE 8 - *Survey of genes involved in cellulose biosynthesis and deposition based on MaizeWall, TIGR, and MGSP (Maize Genome Sequencing Project) databases.*

Gene	bin	MGSP contig	Contig position	MGSP clone and gene
Cellulose synthase (CesA)				
Cellulose synthase-1 (CesA-1)	8.03	ctg344	87.6 Mb	AC198758.3_FG027
Cellulose synthase-2 (CesA-2)	6.05	ctg281	122.0 Mb	AC216196.3_FG032
Cellulose synthase-3 (CesA-3)	3.04	ctg112	10.8 Mb	AC229991.1_FG018
Cellulose synthase-4 (CesA-4)	7.02	ctg297	16.9 Mb	AC205128.3_FG038
Cellulose synthase-5 (CesA-5)	1.11	ctg64	279.7 Mb	AC200189.3_FG038
Cellulose synthase-6 (CesA-6)	1.11	ctg65	285.1 Mb	AC196125.3_FG034
Cellulose synthase-7 (CesA-7)	7.02	ctg300	32.1 Mb	AC195842.3_FG042
Cellulose synthase-8 (CesA-8)	7.02	ctg299	24.3 Mb	AC207393.3_FG035
Cellulose synthase-9 (CesA-9)	2.06	ctg91	152.5 Mb	AC209924.3_FG032
Cellulose synthase 10 (CesA10)	1.08	ctg47	222.4 Mb	AC196799.3_FG040
Cellulose synthase 11 (CesA11)	3.07	ctg141	189.5 Mb	AC203900.3_FG055
Cellulose synthase 12 (CesA12)	7.02	ctg313	94.8 Mb	AC210802.2_FG045
FRA1-like1 “kinesin motor region”	7.02	ctg301	41.0 Mb	AC199397.4_FG035
FRA2-like1 “AAA ATPase”	3.08	ctg145	199.2 Mb	AC210608.3_FG032
FRA2-like2 “AAA ATPase”	8.05	ctg358	143.1 Mb	AC196391.4_FG026
Brittle stalk 2 COBRA-like	9.04	ctg385	90.2 Mb	AC196408.3_FG030
β-1,4-endoglucanase KORRIGAN-like	5.02	ctg207	8.6 Mb	AC195684.3_FG027

lose interactions that maintain organ flexibility rather than having a direct role in cellulose biosynthesis, even if the cellulose content was reduced in mutant plants (Sindhu *et al*., 2007). Supporting data is expected from studies of other rice brittle mutants which are similarly altered in cellulose deposition in the cell wall or in cellulose synthesis (Xu and Messing, 2008). In addition, KORRIGAN mutants have irregular xylem and the corresponding encoded protein is supposed to have a role in processing of the growing cellulose microfibrils or release of the cellulose synthase complex (Szyjanowicz *et al*., 2004).

Genes involved in regulation of phenylpropanoid biosynthesis and deposition

Genes involved in the regulation of monolignol biosynthesis have been described in different species, but little is currently known for maize or grasses. The regulation of phenylpropanoid biosynthesis was the first role identified for a plant R2R3-MYB transcription factor (Paz-Ares *et al*., 1987), which was first illustrated by the heavily reduced lignin content in mature parts of tobacco plants over-expressing an *Antirrhinum* MYB factor (Tamagone *et al*., 1998). R2R3-MYB genes recognize AC cis-regulating elements which are present in promoters of numerous phenylpropanoid genes (Sablowski *et al*., 1994; Peter and Neale, 2004), even if other interaction mechanisms likely exist (Uzal *et al*., 2008). Other pathways are also regulated by R2R3-MYB, and based on aspen data, only 23 out of 180 R2R3-MYB encoding genes showed the highest level of transcript abundance in differentiating xylem (Wilkins *et al*., 2009). Several MYB genes were thus described in maize (Franken *et al*., 1994; Guillaumie *et al*., 2007b), but only ZmMYB31 and ZmMYB42 were 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). ZmMYB31 had a stronger repressing effect on COMT expression than ZmMYB42, but ZmMYB42 also negatively regulated the expression of several genes of the lignin pathway. Moreover, the over-expression of the ZmMYB42 gene in Arabidopsis plants generated a lignin polymer with a decreased S/G ratio due to a lower content in S units (Sonbol *et al*., 2009). Several other MYB factors are surely involved in the regulation of maize lignification. Three MYB genes of barley, associated with vascular bundles (Wissenbach *et al*., 1993), thus have orthologs in maize including the ZmMYB31 and ZmMYB42 genes (Table 9). In addition, at least

TABLE 9 - *Survey of MYB transcription factors putatively involved in the regulation of cell wall component biosynthesis based on MaizeWall, TIGR, MGSP (Maize Genome Sequencing Project) databases, and* ZHONG et al. *(2008).*

Gene	bin	MGSP contig	Contig position	MGSP clone and gene
ZmMYB31 hv5- and EgMYB1-like	2.07	ctg101	187.2 Mb	AC192225.2_FG040
ZmMYB42 hv5-like	3.05	ctg127	135.2 Mb	AC209159.3_FG040
ZmMYB hv5- and EgMYB1-like1	7.04/05	ctg325	151.8 Mb	AC215912.3_FG049
ZmMYB hv5-like2	3.06	ctg133	165.1 Mb	AC209027.3_FG034
ZmMYB hv5-like3	6.06	ctg287	143.7 Mb	AC188838.3_FG025
ZmMYB hv5-like4	1.07	ctg43	193.9 Mb	AC215269.2_FG028
ZmMYB hv5-like6	8.03	ctg345	93.8 Mb	AC209081.3_FG037
ZmMYB AtMYB46-like1	8.03	ctg333	41.7 Mb	AC200623.4_FG039
ZmMYB AtMYB46-like2	9.03	ctg373	32.2 Mb	AC211740.3_FG045
ZmMYB AtMYB46-like3	7.03	ctg321	126.8 Mb	AC184048.4_FG038
ZmMYB AtMYB52-like	8.01	ctg326	3.0 Mb	AC217844.3_FG018
ZmMYB AtMYB58-like1	6.01	ctg265	31.9 Mb	AC196101.2_FG040
ZmMYB AtMYB63-like1	4.06	ctg181	154.6 Mb	AC186596.3_FG032
ZmMYB AtMYB69/54-like1	3.04	ctg117	41.8 Mb	AC192228.3_FG026
ZmMYB AtMYB85-like1	4.06	ctg182	163.7 Mb	AC185471.3_FG047
ZmMYB AtMYB85-like2	7.02	ctg310	86.4 Mb	AC195943.2_FG032
ZmMYB AtMYB85/32-like	2.05	ctg89	130.6 Mb	AC205519.3_FG030
ZmMYB AtMYB103-like1	10.04	ctg413	108.9 Mb	AC198506.3_FG035
ZmMYB AtMYB103/20-like1	4.08	ctg188	201.2 Mb	AC226216.1_FG044
ZmMYB AtMYB103/46-like1	3.05	ctg125	125.2 Mb	AC193497.3_FG023
ZmMYB MaizeWall QAS2H09.yg.3.5	8.05	ctg354	120.8 Mb	AC197007.3_FG036
ZmMYB MaizeWall QAS2H09.yg.3.5	10.03	ctg406	71.4 Mb	AC191417.3_FG025
ZmMYB MaizeWall QBTB.064D08F	5.04	ctg234	133.8 Mb	AC204298.3_FG047

two of these genes are orthologs of EgMYB1 which was shown to be a negative regulator of lignin gene expression (LEGAY *et al.*, 2007) similarly to ZmMYB31 and ZmMYB42, while EgMYB2 was shown to be a transcriptional activator (GOICOECHEA *et al.*, 2005).

The transfer of data and knowledge related to tissue patterning and lignification from dicotyledons or gymnosperms to grasses is difficult due the vascular specific traits in grasses. In contrast to dicotyledonous plants, the vascular system of non-woody monocotyledons is characterized by the absence of bifacial cambium and secondary growth. Monocotyledon 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 thus been considered to have no homology with the dicotyledons vascular system (TERASHIMA and FUKUSHIMA, 1993; TOMLINSON, 1995). However, emergence and evolution of lignified tracheids and vascular tissues was indeed based on the expression of a preexisting poly-phenolic pathway (BOYCE *et al.*, 2003), with lignin targeted deposition in different cell types. In addition, numerous results obtained in genetics and genomics of lignification illustrated a large commonality in cell wall carbohydrate and phenolic biosynthesis in all plants. Orthologs of MYB factors regulating lignin-related gene expression in woody species are therefore likely candidate genes for regulation of maize lignin biosynthesis and deposition, even if the targets have possibly changed during plant evolution, with the possible selection of grass specific genes, as it has been considered for genes involved in arabinoxylan biosynthesis.

A non-exhaustive list of major maize MYB genes involved in cell wall gene regulation can be considered based on well-known MYB genes of woody species (including Arabidopsis), even if a more

TABLE 10 - *Survey of NAC transcription factors putatively involved in the regulation of cell wall phenolic and carbohydrate biosynthesis based on MaizeWall, TIGR, MGSP (Maize Genome Sequencing Project) databases, and* ZHONG et al. *(2008).*

Gene	bin	MGSP contig	Contig position	MGSP clone and gene
NAC SND1/NST1-like1	4.05	ctg164	38.9 Mb	AC209405.3_FG028
NAC SND1/NST1-like2	9.03	ctg373	28.8 Mb	AC204379.3_FG028
NAC SND1/NST1-like3	6.04	ctg276	93.7 Mb	AC211685.4_FG043
NAC SND1/NST1-like4	5.04	ctg235	140.9 Mb	AC187874.3_FG035
NAC SND1/NST1-like5	4.06	ctg179	142.7 Mb	AC216878.3_FG025
NAC VND6-like-1	9.03	ctg373	33.4 Mb	AC216802.3_FG040
NAC VND6-like-2	2.03	ctg74	22.0 Mb	AC212974.3_FG041
NAC VND6-like-3	1.01	ctg3	5.4 Mb	AC217946.3_FG043
NAC VND6-like-4	4.06	ctg179	139.5 Mb	AC208029.3_FG031
NAC VND6-like-5	9.07	ctg391	119.2 Mb	AC204746.3_FG045
NAC VND7-like1	1.01	ctg3	4.7 Mb	AC191725.3_FG047
NAC VND7-like2	2.03	ctg74	25.2 Mb	AC218093.2_FG031
NAC VND7-like3	6.00	ctg256	0.7 Mb	AC196640.3_FG037
NAC SND2/SND3-like1	3.08	ctg146	201.4 Mb	AC217265.3_FG021
NAC SND2/SND3-like2	6.07	ctg289	157.3 Mb	AC204859.3_FG042
NAC SND2-like	2.8/09	ctg107	217.6 Mb	AC201894.4_FG040
KNAT7-like1	1.01	ctg3	5.0 Mb	AC194428.3_FG044
KNAT7-like2	9.07	ctg391	120.2 Mb	AC197598.4_FG051

complete list should be established from extensive investigations such as in RENGEL *et al.* (2009). Among all MYB genes, the orthologous genes EgMYB2 (GOICOECHEA *et al.*, 2005), PtMYB4 (PATZLAFF *et al.*, 2003), PttMYB21 or PtrMYB021 (KARPINSKA *et al.*, 2004; WILKINS *et al.*, 2009), and AtMYB46 (ZHONG *et al.*, 2007) were strongly shown to be regulators of lignin-related genes. The EgMYB2 gene of eucalyptus thus encoded a transcriptional activator binding specifically *cis*-regulatory regions of EgCCR and EgCAD2 genes (GOICOECHEA *et al.*, 2005). The three first maize orthologs of this EgMYB2-type MYB have been considered as putative candidates in maize (Table 9). Other lignin-related MYB genes have been described in aspen (WILKINS *et al.*, 2009), pine (BOMAL *et al.*, 2008) and Arabidopsis (ZHONG *et al.*, 2007, 2008; ZHOU *et al.*, 2009). The corresponding MYB maize orthologs have been searched for mainly based on the two last investigations in Arabidopsis (Table 9). In addition, three other maize MYB genes have been described in the MaizeWall database, including the ZmMYB QBTB.064D08F which was under-expressed in bm1 and bm2 maize young plants (GUILLAUMIE *et al.*, 2007a). This last MYB gene is located 30 Mb, but only 3 cM, downstream the ZmCAD2 gene and is therefore a putative candidate for the bm1 mutation.

Lignin pathway genes are regulated upstream the MYB transcription factor level. NAC proteins were thus shown to be regulators of the expression of several transcription factors involved in secondary wall biosynthesis, including MYB and other NAC Arabidopsis genes (ZHONG and YE, 2007; YAMAGUCHI *et al.*, 2008; ZHONG *et al.*, 2008; ZHOU *et al.*, 2009). Over-expression of NST1, NST2 and SND1 thus induced ectopic lignified secondary cell wall thickenings in various Arabidopsis tissues (MITSUDA *et al.*, 2005, 2007; KO *et al.*, 2007; ZHONG *et al.*, 2006). "Master" NAC genes are activators of the entire secondary wall biosynthetic programs in different cell type, such as SND and NST1 in fibers and VND6 and VND7 in vessels and they regulate other MYB and NAC target genes (YAMAGUCHI *et al.*, 2008; ZHONG *et al.*, 2008). Thirteen putative orthologs of SND1, NST1, VND6, and VND7 were thus shown (Table 10), but their possible regulatory role in maize secondary wall lignification and biosynthesis is not yet established. In addition, putative or-

TABLE 11 - *Survey of transcription or regulation factors putatively involved in the regulation of cell wall component biosynthesis based on MaizeWall, TIGR, and MGSP (Maize Genome Sequencing Project) databases.*

Gene	bin	MGSP contig	Contig position	MGSP clone and gene
ATHB-8 HD-ZIPIII QBN21E06.xg.	1.07	ctg46	218.3 Mb	AC209904.3_FG031
ATHB-8 HD-ZIPIII	1.08	ctg52	241.8 Mb	AC200187.4_FG026
ATHB-8 HD-ZIPIII	9.07	ctg391	121.2 Mb	AC204385.3_FG024
ATHB-8 HD-ZIPIII	3.04	ctg123	107.5 Mb	AC194956.3_FG031
ZmLim1 Ntlim1-like	6.01	ctg261	10.6 Mb	AC202978.3_FG041
ZmLim2 Ntlim1-like	3.05	ctg124	119.4 Mb	AC182621.3_FG033
ZmLim3 MaizeWall QBJ4h04.xg.2	2.03	ctg74	22.5 Mb	AC186516.3_FG039
IFL1 HD-ZIP	1.01	ctg2	2.9 Mb	AC217357.3_FG027
IFL1 HD-ZIP	9.07	ctg391	121.2 Mb	AC204385.3_FG024
Zinc finger C2H2 EucaWood ctg6994	8.05	ctg354	127.9 Mb	AC198977.3_FG040
ZmZAG5 (Shatterproof MADS box)	4.06	ctg181	153.0 Mb	AC203944.4_FG022
COV1-like1	3.06	ctg134	167.1 Mb	AC217263.3_FG020
COV1-like2	6.06	ctg288	153.9 Mb	AC206165.3_FG045
COV1-like3	8.05	ctg353	116.5 Mb	AC206497.2_FG041
COV1-like4	8.07	ctg363	158.5 Mb	AC194935.3_FG036
Argonaute MaizeWall 131537.2.170	6.07/08	ctg291	161.1 Mb	AC205572.3_FG040

thologs of their targets were identified in maize with three SND2/SND3 NAC genes and two KNAT7 homeobox protein knotted-1-like7 encoding genes.

Zinc-finger proteins constitute one of the largest families of transcription factor regulatory proteins, and class III homeodomain leucine-zipper (HD-ZIP) proteins have been shown to play a regulatory role in vascular differentiation. 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 in lignin content with an ectopic lignin distribution, or a nearly 40% decrease in lignin content with a decrease in the S/G ratio, respectively (Sanchez *et al*., 2006). Tran *et al.* (2006) also reported the interaction between NAC and zinc finger homeodomain proteins. Based on the MaizeWall database, four ATHB-8 HD-ZIPIII orthologous genes putatively involved in maize lignification were found (Table 11). In addition, members of the LIM protein family that contain a zinc finger motif were shown to be regulators of lignin genes. Transgenic tobacco plants with antisense Ntlim1 thus showed low levels of transcripts from several key phenylpropanoid pathway genes such as PAL, 4CL and CAD. Transgenic tobacco plants with antisense Ntlim1 gene also had a greater than 20% reduction in lignin content (Kawaoka and Ebinuma, 2001). Two orthologs of the NtLim1 gene were found in the maize genome (Table 11), which were different from the Lim gene under-expressed in maize bm1 and bm2 plantlets (Guillaumie *et al*., 2007a). The INTERFASCICULAR-FIBERLESS-1 (IFL1) gene, which is also a member of the plant homeodomain-leucine zipper family, regulates interfascicular fiber differentiation and stem strength in Arabidopsis (Zhong and Ye, 1999; Ratcliffe *et al*., 2000). Two orthologs of this gene were found in the maize genome (Table 11). Zinc-finger C2H2 genes were the most frequently represented transcription factors in eucalyptus secondary xylem libraries (EucaWood database; Rengel *et al*., 2009), including one gene with an identified rice ortholog (Table 11), while no zinc-finger C2H2 genes had been previously described in maize lignifying tissue (MaizeWall database; Guillaumie *et al*., 2007b).

Other regulating factors are probably involved in the biosynthesis and deposition of phenolic compounds in the maize cell wall. The SHP1 (SHATTERPROOF MADS-box) gene, which specifies with

SHP2 the lignified valve margin of mature Arabidopsis siliques (LILJEGREN *et al*., 2000), has for an ortholog in maize the ZmZAG5 gene, the function of which is not known. However, in young stems of the bm2 mutant, the maize ZmZAG5 gene was under-expressed to nearly the same level as the disrupted COMT gene in bm3 plants, while it was 3.5 times over-expressed in the ear internode of bm3 silking plants (BARRIÈRE *et al*., 2004c; GUILLAUMIE *et al*., 2007a, 2008). The COV1 (CONTINUOUS VASCULAR RING) recessive mutant of Arabidopsis has a great increase in stem vascular tissue at the interfascicular regions (PARKER *et al*., 2003). The COV1 mutant is defective in synthesis, transport, or perception of a vascular patterning inhibitor by a non auxin-dependant mechanism. One of the COV1 maize orthologs, located in bin 8.03, was 3.0 times over-expressed in bm3 ear lignifying internodes (GUILLAUMIE *et al*., 2008), thus possibly illustrating an unknown form of inhibition in lignified tissue formation (Table 11).

The role of micro-RNA in regulation of plant lignified tissue assembly is little documented. In aspen, an HD-Zip transcription factor (PtaHB1), closely associated with wood formation, and a miRNA (Pta-miR166) have inversely correlated expression levels (KO *et al*., 2006). The maize ZmmiR166 miRNA accumulates in phloem and regulates the maize rolled-leaf1 gene (rld1), which encodes an HD-ZIP III transcription factor. The Arabidopsis ortholog is the IFL1/REVOLUTA gene, which is involved in the differentiation of interfascicular fibers and secondary xylem (RATCLIFFE *et al*., 2000; JUAREZ *et al*., 2004). Post-transcriptional regulation via miRNA-directed cleavage was also shown for several, but not all, NAC genes (LAUFS *et al*., 2004; GUO *et al*., 2005; YAMAGUCHI *et al*., 2008). In addition, small interfering RNA derived from the3'-coding region of CesA6 cellulose synthase of barley were shown to be involved in the transition from primary to secondary cell wall programs (HELD *et al*., 2008). Among genes required for post-transcriptional gene silencing and RNA interference, at least the member of the Argonaute family, which was over-expressed in bm3 and AS225 deficient COMT plants, is a possible candidate to be involved with HD-ZIP III transcription factors in the regulation of maize lignification (Table 11).

STRATEGIES FOR RELEVANT CANDIDATE GENE DISCOVERY AND VALIDATION

While much data is available concerning genes involved in the maize lignin pathway, there are still many unanswered questions about maize cell wall biosynthesis and assembly. The respective roles of each member in each multigene family are not really known, nor are their respective expression kinetics in each tissue during plant growth. The transcription

TABLE 12 - *Comparative expression of phenylalanine ammonia lyase (PAL), caffeoyl-CoA O-methyltransferase (CCoAOMT), caffeic acid O-methyltransferase (COMT), and cinnamyl alcohol dehydrogenase genes in ear internode of silking maize plants, in F2bm3 and F2 line (data from GUILLAUMIE* et al*., 2008), in F268 and F286 (data from BARRIÈRE* et al*., 2007, 2009), in Fl222 and F2 (unpublished data from Génoplante network), and in RIL99 and RIL39 (data from THOMAS* et al. *2009) Lines cell wall traits are given in table 1. For each of the three comparisons, normalized expression values are given for lines with the lower cell wall digestibility and ratios of signal intensity between lines of high and low cell wall degradability. Genes were considered as significantly differentially expressed when expression ratio values were lower than 0.5 or higher than 2.0 (GUILLAUMIE* et al., *2007a).*

	F2	F2bm3/F2	F268	F286/F268	Fl222	F2/Fl222	RIL39	RIL99/RIL39
PAL (pal3a)	187353	0.22	31307	0.70	158123	1.20	201934	0.37
PAL (pal3b)	207907	0.44	57310	0.31	154642	1.34	36216	0.43
PAL (pal2a)	102659	0.90	116420	0.26	221058	0.46	96523	0.34
CCoAOMT1	21976	0.88	9657	0.77	110003	0.20	29928	1.07
CCoAOMT2	47434	0.77	13612	0.74	46807	1.01	83772	1.23
CCoAOMT3	23740	3.18	18453	0.82	48638	1.32	21622	1.42
CCoAOMT4	13947	2.38	7924	0.79	17838	0.78	13538	1.37
CCoAOMT5	51550	1.01	71160	0.39	156905	0.33	55910	1.77
COMT	142203	0.05	81210	0.36	407362	0.35	260901	0.44
ZmCCR1	37894	0.80	18662	0.83	127228	0.29	51858	0.52
ZmCAD2	30285	0.80	42403	0.35	132867	0.23	83317	0.33

factors with major effects on cell wall lignification and degradability are not known despite despite the fact that this is a key-step for future progress in silage and biofuel maize breeding. Regulating factors involved in lignified tissue assembly and tissue patterning have not yet been established despite idespite the fact this is strategic in breeding maize with both high cell wall degradability and high stalk standability. How ferulate is synthesized and how linkages to arabinoxylan are driven has not yet been determined while it is likely one basis of cell wall degradability and stiffness. In addition, it is still not clear how monolignols and FAXX are transported from the cytosol to the cell wall, which is the basis of compound availability in the cell wall.

In search of candidate genes from expression studies

Besides comparative studies of normal and brown-midrib maize lines, a few investigations have compared gene expression in lines with variable ranges of lignin content and/or cell wall degradability (Barrière *et al*., 2007, 2009; Shi *et al*., 2007; Guillaumie *et al*., 2008; Thomas *et al*., 2009), mostly based on the MaizeWall macro-array (Guillaumie *et al*., 2007b). In this set of limited experiments, with investigations of gene expression in ear internode at silking stage, lines with higher cell wall degradability appeared to be characterized by lower expression of at least one PAL gene and simultaneous lower expression of one or several genes in the monolignol pathway (Table 12). However, the lower expression of several phenylpropanoid genes in lines of higher cell wall degradability is more probably related to a variable expression of a transcription factor rather than to allelic variation of one or more lignin genes, with pleiotropic consequences on other ones (such a co-regulation was however observed in the normal / bm3 comparison). More information will be obtained with expression studies based on micro-array with a larger set of genes and regulation factors. Moreover, variations in gene expression may also be related to variation in extent of lignified tissue assembly, rather than true gene expressions in each tissue. Such variation could correspond to variable expression of regulatory genes, possibly also including genes related to auxin-regulated patterning mechanisms.

In search of candidate genes from QTL analyses

Lignification trait QTL are available from several maize RIL progenies, developed either for cell wall digestibility or for corn borer tolerance studies (Cardinal *et al*., 2003; Krakowsky *et al*., 2004, 2005; Roussel *et al*., 2002; Méchin *et al*., 2001; Barrière *et al*., 2008). Nearly 40 locations were thus shown with hot spots in bins 1.07, 2.07/08, 3.01, 3.04, 5.03/04, and 6.05/06 (Barrière *et al*., 2007). Based on four RIL progenies, QTL for cell wall digestibility (IVNDFD) were shown in nearly 20 locations (*per se* value experiments) out of which there were 13 locations with R^2 values higher than 10% (Table 13). QTL for IVNDFD and lignin content appears to be colocalizing in nearly 75% of occurrences, but 45% of lignin content QTL do not colocalized with IVNDFD QTL. Variations in lignin structure and cross-linkages between arabinoxylan chains and guaiacyl units of lignins, or between arabinoxylan chains, likely explain the part of cell wall degradability variations not explained by lignin content variations.

Colocalizations between lignin or IVNDFD QTL and genes involved in monolignol biosynthesis were searched for based on physical positions of genes and putative QTL. While this first approach must be considered cautiously because QTL physical position are approximative and because results are greatly dependent on the set of investigated genes, the most frequent associations are observed between IVNDFD and/or lignin QTL and NAC or MYB genes. In some cases, several genes of the monolignol pathway are also observed in the same locations (Table 14). MYB factors ZmMYB hv5-like4 and like5 in bin 1.07 and 6.06, and NAC SND1/NST1-like1 and -like2 in bin 4.05 and 9.02, are probable candidates underlying cell wall digestibility QTL.

In search of candidate genes from association genetic studies

During the last decade, association genetics (or association mapping) emerged as a complementary approach to identify polymorphisms involved in phenotypic variation and alleles of interest for agronomic purposes. As opposed to QTL mapping, this method takes advantage of existing collections and historical/evolutionary recombination to study the correlation between large genetic diversity and phenotypic variation, with the screen of a larger diversity and a higher resolution due to the accumulation of much more recombination events. In maize, since the pioneer work of Thornsberry *et al.* (2001) who explored the associations between flowering time (FT) and a suite of polymorphisms

TABLE 13 - *Putative QTL for ADL/NDF, IVNDFD, and* p*-hydroxycinnamic acids with lod > 2.5 in four recombinant inbred line progenies experimented in per se value (IVNDFD = in vitro NDF digestibility with NDF = neutral detergent fiber; ADL = acid detergent lignin;* p*-Coumaric acid (*p*Ca), etherified ferulic acids (etherFA) released after alkaline hydrolysis. distance is given as cM). Data from QTL analyses in RIL Io x F2 (*Méchin* et al*., 2001), F288 x F271 (*Roussel* et al*., 2002; *Thomas* et al*., 2009), F7025 x F4 (Génoplante unpublished data), and F838 x F286 (*Barrière* et al*., 2008a).*

RIL progeny	Trait	chr-pos	bin	Closest marker	Marker position	QTL appr. position	Lod	R^2
F838 x F286	ADL/NDF	1-56	1.01	bnlg1112	9.8 Mb	8.1 Mb	3.5	6.4
F838 x F286	ADL/NDF	1-76	1.02	bnlg1178	14.0 Mb	15.3 Mb	4.6	8
F838 x F286	IVNDFD	1-80	1.02	bnlg1178	14.0 Mb	17.8 Mb	4.8	9
F838 x F286	*p*CA	1-80	1.02	bnlg1178	14.0 Mb	17.8 Mb	6.7	12
F838 x F286	EtherFA	1-82	1.02	bnlg1178	14.0 Mb	19.0 Mb	6.5	12
F288 x F271	IVNDFD	1-74	1.02	bnlg1627	18.6 Mb	26.9 Mb	2.7	9
F7025 x F4	ADL/NDF	1-76	1.03	bnlg2238	54.2 Mb	50.2 Mb	9.5	17
F7025 x F4	IVNDFD	1-78	1.04	bnlg2238	54.2 Mb	52.3 Mb	5.7	11
F838 x F286	ADL/NDF	1-162	1.05	bnlg1832	79.0 Mb	83.0 Mb	5.0	9
F838 x F286	IVNDFD	1-160	1.05	bnlg1832	79.0 Mb	83.0 Mb	3.6	7
F838 x F286	EtherFA	1-154	1.05	bnlg1832	79.0 Mb	83.0 Mb	5.3	10
F838 x F286	*p*CA	1-160	1.05	bnlg1832	79.0 Mb	83.0 Mb	7.5	13
F838 x F286	EtherFA	1-224	1.07	bnlg1556	196.2 Mb	190.2 Mb	5.2	9
F838 x F286	ADL/NDF	1-228	1.07	bnlg1556	196.2 Mb	198.0 Mb	3.3	6
F838 x F286	IVNDFD	1-228	1.07	bnlg1556	196.2 Mb	198.0 Mb	6.7	12
F838 x F286	*p*CA	1-228	1.07	bnlg1556	196.2 Mb	198.0 Mb	6.0	11
F838 x F286	ADL/NDF	2-14	2.02	bnlg1302	9.6 Mb	9.4 Mb	3.1	6
F7025 x F4	IVNDFD	2-90	2.02	bnlg381	27.7 Mb	15.7 Mb	2.6	5
F7025 x F4	ADL/NDF	2-102	2.02	bnlg381	27.7 Mb	23.7 Mb	3.1	6
F838 x F286	EtherFA	2-88	2.06	bnlg1138	164.2 Mb	139.2 Mb	3.2	6
F7025 x F4	ADL/NDF	2-156	2.07	bnlg1045	191.0 Mb	196.0 Mb	3.0	6
F838 x F286	ADL/NDF	2-182	2.08	bnlg1940	209.4 Mb	206.8 Mb	4.6	8
F838 x F286	IVNDFD	3-44	3.04	bnlg1904	9.7 Mb	46.3 Mb	2.9	5
F838 x F286	*p*CA	3-58	3.04	umc1425	15.6 Mb	55.6 Mb	3.7	7
F838 x F286	ADL/NDF	3-66	3.04	umc1773	103.7 Mb	63.7 Mb	7.5	13
F838 x F286	EtherFA	3-70	3.04	umc1773	103.7 Mb	63.7 Mb	11.4	20
F288 x F271	ADL/NDF	3-110	3.05/06	bnlg1505	145.2 Mb	146.3 Mb	2.2	7
F838 x F286	*p*CA	3-100	3.06	umc1311	161.8 Mb	154.8 Mb	3.3	6
F838 x F286	IVNDFD	4-66	4.05	bnlg1265	44.1 Mb	39.6 Mb	7.5	13
F838 x F286	EtherFA	4-66	4.05	bnlg1265	44.1 Mb	39.6 Mb	4.6	9
Io x F2	ADL/NDF	4-92	4.05	sc315s	-	-	2.4	9
F7025 x F4	ADL/NDF	4-72	4.06	bnlg252	45.7 Mb	63.7 Mb	7.3	14
F7025 x F4	IVNDFD	4-76	4.06	bnlg252	45.7 Mb	71.7 Mb	6.2	12
F7025 x F4	ADL/NDF	4-128	4.08	bnlg2162	189.6 Mb	191.0 Mb	3.2	6
F7025 x F4	IVNDFD	4-136	4.08	bnlg2162	189.2 Mb	199.2 Mb	7.0	13
F7025 x F4	ADL/NDF	5-58	5.03	bnlg1046	17.7 Mb	43.6 Mb	2.8	5
F838 x F286	ADL/NDF	5-106	5.06	bnlg1847	183.4 Mb	180.9 Mb	3.6	7
F7025 x F4	ADL/NDF	6-92	6.04	nc012	139.2 Mb	120.2 Mb	5.2	10
F7025 x F4	IVNDFD	6-92	6.04	nc012	139.2 Mb	120.2 Mb	3.3	6.3
F288 x F271	ADL/NDF	6-142	6.06	bnlg345	146.6 Mb	149.8 Mb	6.5	20
F288 x F271	IVNDFD	6-142	6.06	bnlg345	146.6 Mb	149.8 Mb	14.4	40

TABLE 13 - *Continued*.

RIL progeny	Trait	chr-pos	bin	Closest marker	Marker position	QTL appr. position	Lod	R^2
F838 x F286	ADL/NDF	7-70	7.02	bnlg1808	102.4 Mb	106.4 Mb	5.0	9
Io x F2	IVNDFD	7-36	7.03	umc116a	103.9 Mb	-	3.3	11
F7025 x F4	IVNDFD	7-28	7.03	bnlg1305	107.8 Mb	108.8 Mb	2.5	5
F838 x F286	EtherFA	8-46	8.03	bnlg1834	52.7 Mb	81.7 Mb	5.6	10
F7025 x F4	ADL/NDF	8-88	8.05/06	bnlg1782	148.2 Mb	128.3 Mb	7.3	14
F7025 x F4	IVNDFD	8-96	8.05/06	bnlg1782	148.2 Mb	146.2 Mb	3.0	6
F838 x F286	EtherFA	8-118	8.07	bnlg1065	157.1 Mb	172.0 Mb	5.0	9
F838 x F286	*p*CA	8-120	8.07	bnlg1065	157.1 Mb	176.2 Mb	2.9	5
F838 x F286	ADL/NDF	8-150	8.07	bnlg1065	157.1 Mb	212.0 Mb	4.5	8
F838 x F286	IVNDFD	8-134	8.07	bnlg1065	157.1 Mb	210.5 Mb	5.8	10
F288 x F271	IVNDFD	9-52	9.02	bnlg1401	24.2 Mb	24.0 Mb	4.0	13
F288 x F271	ADL/NDF	9-62	9.02	bnlg1401	24.2 Mb	25.2 Mb	4.0	13
F7025 x F4	ADL/NDF	9-124	9.06	bnlg1191	111.7 Mb	110.4 Mb	6.3	12
F7025 x F4	IVNDFD	9-126	9.06	umc1310	113.7 Mb	113.7 Mb	3.6	7.0
F838 x F286	IVNDFD	10-30	10.02	umc1337	6.4 Mb	24.4 Mb	13.7	23
F838 x F286	*p*CA	10-30	10.02	umc1337	6.4 Mb	24.4 Mb	6.6	12
F838 x F286	ADL/NDF	10-50	10.04	umc1246	82.2 Mb	78.2 Mb	9.4	17
F838 x F286	EtherFA	10-54	10.04	umc1246	82.2 Mb	86.2 Mb	4.4	8

in the transcription factor DWARF8, the method has been widely extended to many other candidate gene polymorphisms and phenotypic variation. A crucial aspect of association genetics is the knowledge of linkage disequilibrium (LD) i.e. non independent association of unrelated loci. In maize, LD has been shown to decrease fast with physical distance within genes (TENAILLON *et al*., 2001; REMINGTON *et al*., 2001; CHING *et al*., 2002), precluding of a high resolution of the method (RAFALSKI, 2002). However, several studies suggested that recombination may strongly differ within and between maize genes, since LD nearby candidate genes may persist much longer than within genes, i.e. along 100kb around ADH1 (JUNG *et al*., 2004) and 600kb upstream Y1 (PALAISA *et al*., 2003). Additionally, polymorphisms shown to be responsible for phenotypic variation were located in regulatory rather than coding regions, sometimes several tens of kb away from the candidate gene itself, as illustrated by TB1 (CLARK *et al*., 2005) or VGT1, a cis-acting regulatory region located 70 kb upstream the APETALA2-like gene (SALVI *et al*., 2007; DUCROCQ *et al*., 2008). The first maize association mapping results thus highlight the importance of regulatory variation, and suggest that candidate gene polymorphism and local LD studies should be extended to candidate regions that include cis-regulatory sequences.

Additionally, genome-wide LD that results from population stratification can generate non-functional, spurious associations. In maize, intense stratification results from genetic drift during geographical expansion and increased relatedness among elite material derived by plant breeders (DOEBLEY *et al*., 1988; CAMUS-KULANDAIVELU *et al*., 2006; VIGOUROUX *et al*., 2008). Controlling for LD due to population stratification is thus a major issue. The most consensual statistical method in plant studies is based on the inference of population structure (PRITCHARD *et al*., 2000a) and individual kinship (RITLAND, 1996; ZHAO *et al*., 2007), both from random molecular markers across the genome, and their use in association analyses through linear regression (for population control alone, PRITCHARD *et al*., 2000b) or a mixed model (to control for both population structure and individual kinship, YU *et al*., 2006). Both models are implemented in the TASSEL software (BRADBURY *et al*., 2007). In their study of DWARF8 - flowering date association mapping, THORNSBERRY *et al*. (2001) revealed that taking population structure into account reduced the number of false positive associations by

TABLE 14 – *Colocalizations between cell wall related trait QTL, monolignol biosynthesis pathway, and transcription factors in seven hop-spot locations.*

Genes and QTL	bin	Position	Genes and QTL	bin	Position
ZmMYB hv5-like4	1.07	193.9 Mb	ZmLac3	4.08	187.7 Mb
4CL2	1.07	197.7 Mb	QTL ADL/NDF (F7025 x F4)	4.08	191.0 Mb
QTL ADL/NDF (F838 x F286)	1.07	198.0 Mb	OMT ZRP4-like4	4.08	197.0 Mb
QTL IVNDFD (F838 x F286)	1.07	198.0 Mb	CCoAOMT4	4.08	198.8 Mb
QTL *p*CA (F838 x F286)	1.07	198.0 Mb	CCoAOMT5	4.08	198.8 Mb
CCR1 (ZmCCR1)	1.07	199.3 Mb	QTL IVNDFD (F7025 x F4)	4.08	199.2 Mb
F5H1	1.07	211.8 Mb	ZmMYB AtMYB103/20-like1	4.08	201.2 Mb
QTL IVNDFD (F7025 x F4)	2.02	15.7 Mb	ZmMYB hv5-like3	6.06	143.7 Mb
QTL ADL/NDF (F7025 x F4)	2.02	23.7 Mb	C3'H2	6.06	148.7 Mb
NAC VND6-like-2	2.03	22.0 Mb	QTL ADL/NDF (F288 x F271)	6.06	149.8 Mb
PAL2a (pal2 locus)	2.03	22.2 Mb	QTL IVNDFD (F288 x F271)	6.06	149.8 Mb
PAL2b (pal2 locus)	2.03	22.2 Mb	OMT ZRP4-like5a	6.06	151.7 Mb
ZmLim3 MaizeWall	2.03	22.5 Mb	OMT ZRP4-like5b	6.06	151.7 Mb
NAC VND7-like2	2.03	25.2 Mb	OMT ZRP4-like5c	6.06	151.7 Mb
			COV1-like2	6.06	153.9 Mb
ZmMYB AtMYB69/54-like1	3.04	41.8 Mb	NAC SND2/SND3-like2	6.07	157.3 Mb
QTL IVNDFD (F838 x F286)	3.04	46.3 Mb			
*p*CA (F838 x F286)	3.04	55.6 Mb	CCoAOMT2	9.02	21.9 Mb
			QTL IVNDFD (F288 x F271)	9.02	24.0 Mb
COMT	4.04	32.7 Mb	QTL ADL/NDF (F288 x F271)	9.02	25.2 Mb
NAC SND1/NST1-like1	4.05	38.9 Mb	HCT3	9.02	26.7 Mb
QTL IVNDFD (F838 x F286)	4.05	39.6 Mb	NAC SND1/NST1-like2	9.03	28.8 Mb
QTL EtherFA (F838 x F286)	4.05	39.6 Mb	ZmMYB AtMYB46-like2	9.03	32.2 Mb
			NAC VND6-like-1	9.03	33.4 Mb

up to 4.7 fold. Providing a sufficient number of neutral markers, the mixed model that includes individual kinship was shown to perform even better and fully remove false positives (YU *et al*., 2006), which is particularly relevant for highly structured phenotypic variation such as flowering date (YU *et al*., 2006), kernel weight (MANICACCI *et al*., 2007), or likely any trait linked to geographical adaptation.

For cell wall related traits, only two association genetics studies are available both with the COMT genes and based on a reduced set of lines. The maize COMT maize gene was shown greatly variable, not only with many SNP and INDEL in its unique intron, but also with several variations in exons leading to several amino acid changes. One INDEL, located in the intron explained 32% (P = 0.0017) of the observed cell wall digestibility variation (GUILLET-CLAUDE *et al*., 2004). Similarly, one INDEL polymorphism within the COMT intron has revealed significant association with stover digestibility in another set of maize lines (LÜBBERSTEDT *et al*., 2005). Complementary studies with a greater number of lines and taking into account the population stratification are needed before more definite conclusions can be drawn.

The great gap in agronomic value between lines of interest for feeding value traits and elite modern lines imposes specific strategies of introgressing feeding value traits in elite germplasm. Association studies between single nucleotide polymorphism (SNP) or insertion - deletion polymorphism (Indel) in cell wall related genes, and cell wall traits, will give functional markers more efficiently used in marker-assisted selection than anonymous markers (ANDERSEN and LÜBBERSTEDT, 2003). Breeding for higher cell wall degradability, based on COMT weak alleles rather than on the null allele is expected to prevent or limit the negative agronomic consequences. However, the association genetics strategy has to be developed without a priori because the key-genes of primary interest for silage and biofuel maize breeders are currently unknown.

Search and validation of candidate genes from transposon tagging and genetic engineering

Genetic engineering and transposon tagging (forward and reverse genetics) are means of discovering and validating the genes involved in lignification and cell wall biosynthesis. Extensive reviews of lignin pathway gene deregulations have investigated the resulting consequences on lignin content and structure in normal and transgenic plants, highlighting gene functions in lignified tissue assembly (Boudet, 2000; Chen *et al*., 2001; Dixon *et al*., 2001; Halpin *et al*., 2004; Davin *et al*., 2008; Ralph *et al*., 2008a).

Three studies of gene down-regulation are devoted to maize, with investigations in COMT deregulated plants (Piquemal *et al*., 2002; He *et al*., 2003; Pichon *et al*., 2006), in addition to all studies devoted to bm3 plants (Barrière *et al*., 2004c). Even if the most important improvements in maize cell wall digestibility have been observed in bm3 plants, COMT down-regulation is perhaps not the best way to improve silage quality or biofuel production. The drawback of COMT down-regulation, or silencing, is the correlative S unit decrease. The aromatic C5 position of G units is free to form C-C resistant linkages, and the 5-OH-G units form resistant benzodioxane structures during lignin polymerization. Lignins of bm3 plants, with a lower S/G ratio, have a relatively greater inhibitory effect than lignins of normal plants (Thorstensson *et al*., 1992). In addition, the COMT deregulation does not impact FA content and ferulate cross-linkages. Finally, altering the expression of appropriate transcription factors involved in lignin pathway gene regulation or tissue patterning regulation could be a better strategy, by affecting a group of target genes in a metabolon in a coordinated manner.

Transposon tagging is a powerful strategy for gene discovery, which is however limited by the large number of plants to be observed for each investigated trait, especially in forward genetics programs when no candidate genes are available. In the framework of cell wall degradability gene discovery, the most efficient way of using the transposon tagging strategy is likely as a functional validation tool. A mutant was thus found in the exon of a G6DH1 gene (Génoplante unpublished data) inducing a reduced content in cell wall content of xylose of (18.0 vs 20.2% cell wall residue) and esterFA (5.9 vs 6.8 mg/g NDF), corroborating the relationships between arabinoxylans and ferulate. This mutant has also for still unknown reasons a lower cell degradability (18.7 vs 20.7% in IVNDFD), but a higher hemicellulose content with a correlative lower cellulose content in the cell wall has been shown in lines of higher cell wall degradability (Riboulet *et al*., 2008). The favorable ideotype is thus expected to have reduced esterFA without reduced hemicellulose contents. A mutant affected in the first intron of the ZmCCR1 was also investigated. A reduced lignin content (4.4 vs 5.1% of ADL/NDF) with an increase in cell wall degradability (23.0 vs 25.3% IVNDFD) was observed simultaneously with an unexpected reduced H content in lignin (1.2 vs 2.2%). The two results both illustrate the complex regulations between genes involved in cell wall assembly and strengthen the probable organization of lignin pathway enzymes as endoplasmic-reticulum-associated multi-enzymes complexes (Winkel, 2004). These enzymatic complexes probably function as different metabolons, each dedicated to the production of the different phenylpropanoid end-compounds (Winkel, 2004). Each member gene in a multigene family could thus be differentially involved in different metabolons, with differential consequences of its deregulation.

Breeding for reduced ferulic acid content in grasses is a primary objectivein term of improving silage quality and biofuel production. From a screening based on biochemical analyses on the expanded first leaf, a transposon tagged mutant was found with reduced etherFA cross-linkages and higher cell wall degradability, without changes in lignin content, in mature stems (Jung and Phillips, 2008). The corresponding gene has not yet been identified, but great advances are expected from this gene discovery.

GENETIC RESOURCES FOR ALLELIC VARIATION SEARCH IN CANDIDATE GENE

The first European-bred flint x dent hybrids rapidly conquered European countries after the introduction of US dent x dent hybrids in the early fifties. Flint lines of early hybrids from the late fifties to the late seventies mostly belonged to the Lacaune and Yellow Bade groups while dent lines were mostly related to the Wisconsin and Minnesota bred germplasms. The registration of Dea (1980) in France and Golda (1984) in Germany illustrated major changes in dent, and to a lesser extent flint, maize germplasm. These varieties marked the beginning of a second era in European maize breed-

ing with genotypes having in most cases lower cell wall degradability. Golda is the model hybrid which had an early BSSS-related line as female, while Dea is the model hybrid which has an Iodent-related line as female. Since Dea registration, the Iodent germplasm has become the foundation of nearly all early hybrids and the basis of further early maize improvements in Europe. Early and medium early dent lines are now related to Iodent and BSSS families, sometimes introgressed of later dent germplasms. Early European flint lines are now often introgressed by dent germplasm, and a few early European hybrids have even two dent parents. As a consequence, while at least two thirds of hybrids registered before 1980 had cell wall degradability higher or equal to that of LG11, this was true for only one eighth of hybrids registered between 1994 and 2002 (BARRIÈRE *et al*., 2004a). The continual decline in the average cell wall digestibility observed since 1980 has now ceased with breeding of specialized silage maize. However, the cell wall degradability of the best modern hybrids does not equal that of old types such as Inra258. Further improvements in maize cell wall digestibility towards silage and biofuel production in the USA or in Europe will require targeted (re)-introduction of original germplasm in currently used elite germplasm.

Most of flint genetic resources with high cell wall degradability are related to a few European and Argentina landraces. Among INRA lines, F4 related to Northern flint germplasm, F7 originating from the Lacaune landrace, F66 related to the Sost landrace, F286 and F324 related to both F7 and Argentina origins are likely resources for most of the favorable traits involved in cell wall degradability (Table 1). These lines are no longer used in modern hybrids, due to their lower combining ability values for yield, and for older ones, stalk rot or lodging susceptibility. In addition, F268, Fl222, and EA1301 are likely resources for identification of major traits related to low cell wall degradability.

Genetic variation for cell wall quality traits is lesser known in dent germplasm, despite the fact that it represents the largest share of available genetic resources in maize. In most cases, BSSS, Iodent, and other dent related modern germplasm lines have rather low cell wall degradability. Out of publicly available early dent lines, W117 and F113 were shown several times to have high cell wall degradability. Other old lines such as F115 and W33 are also suspected to be of interest for this trait (F115 x W33 is the female of Inra258). This fact is also strengthened by the high cell wall degradability value observed in the old hybrid Inra240 (Table 15) because F107 is a progeny from the cross F115 x W33. All these lines have a common ancestry in the old open-pollinated variety Minnesota13, but not all Minnesota13 lines have high cell wall degradability. Several INRA lines have been bred for higher cell wall degradability, based on crosses with F4, F113, and W117, and a few lines are now available for further breeding with improved agronomic traits (Table 1). In addition, high cell wall degradability has been recently shown in the INRA lines F7103

TABLE 15 - *Comparison of cell wall digestibility (NDFD) and agronomic traits in representative registered hybrids across eras of breeding (NDFD* = in vivo *NDF digestibility with NDF = neutral detergent fiber). Given data are adjusted from* BARRIÈRE et al. *(2004a) and French registration experiments. Cell wall degradability and yield values of more recently registered hybrids are given as a non exhaustive illustration of genetic variations and improvements available in current silage maize varieties.*

	Pedigree	Registration year	NDFD (%)	Yield (t/ha)
Inra258	(F115 x W33) x (F7 x Ep1)	1958	54.8	11.7
LG11	(F7 x F2) x W401	1970	50.8	12.7
Funk245	Private	1970	54.8	11.9
Inra240	(F107 x F113) x (F7 x F2)	1972	56.1	12.3
Dea	Private Iodent line x F2	1980	49.3	14.1
Dk250	Private Iodent line x F2	1986	49.5	13.9
Helga	Private	1989	46.4	15.8
Fanion	Private	1991	49.1	15.1
Anjou285	Private	1994	43.3	16.4
Anjou258	Private	1996	48.5	16.2
Best NDFD hybrids	Private	2008	49.0	16.8

related to BSSS and tropical material, and in F7104 related to Tuxpeño germplasm (INRA Lusignan and ProMaïs unpublished data). In later germplasm, dent lines are available from the Wisconsin Quality Synthetic (FREY *et al.*, 2004) such as W94129 and W95115 lines, with lignin contents significantly lower than in lines of similar earliness. Model dent lines of low cell wall degradability are available in a few public lines (Table 1) and in several private Iodent and BSSS lines. The INRA F271 line is characterized by both its great earliness giving FAO 200 hybrids, and its very poor cell degradability possibly related to an intense lignification of its parenchyma cells (MÉCHIN *et al.*, 2005).

The germplasm currently used in maize breeding represents only a small share of the available genetic resources. Most of this germplasm corresponds to resources chosen for grain maize breeding, or to progenies of resources chosen for the latter, even if several breeding companies also have programs devoted to silage and now biofuel purposes. Consequently, it is questionable whether it is of interest to carry out investigations of cell wall traits in unused accessions, old lines, and exotic resources, in order to discover new mechanisms or alleles allowing significant wall degradability improvement. Large genetic variations for lignin content and cell wall digestibility were shown in lines derived from old or unused landraces (INRA and ProMaïs unpublished data). However, it is not yet known if original alleles are specifically present in these newly developed lines.

CONCLUSION

With huge advances in genomics, maize may be considered as the model grass for lignification and degradability studies. At present, similar research efforts are not being conducted on cell wall biosynthesis in other annual or perennial grass forage plants, nor in rice. The availability of the maize genome sequence strengthens its position as a model plant for forage and biofuel production improvement. The short-cycle model plant *Brachypodium distachyon* will be a complementary resource for gene mining and validation.

In the search for a biomass ideotype in maize, it is still open to debate whether breeding efforts should be focused on either biomass yield or rather on biomass degradability. However, a high biomass yield alone could lead to disappointment if cow milk yield is reduced due to lower silage intake and digestibility. Similarly, for biofuel production, low degradable biomass will incur greater transport, processing and fermentation cost. Therefore, there is little doubt that cell wall degradability should be one of the major targets for improvement in the economical value of silage and biofuel grass plants. However, breeding grain maize varieties for the specific ability of their straw to be converted into ethanol is likely not economically possible in the short term period. In grain maize hybrids, the elimination of very unfavorable lignin or cell wall structures should be progressively added as breeding traits for bi-functional grain and straw-ethanol plants.

Among plant polymers, lignins have metabolic plasticity, with variable structures and non-conventional monomer incorporation, giving large variation in cell wall properties for which underlying genetic mechanism are mainly unknown. The lignin composition and structure in modern maize lines and hybrids are the result of long term grass evolution and short term maize breeding efforts towards high yield and biotic or abiotic stress tolerances. To date, the challenge is to change cell wall and lignin polymer properties while keeping the high agronomic value of hybrids. Cheap and sustainable biofuel and silage maize hybrids must be hardy and drought tolerant, capable of enduring and recovering after hot and dry conditions, tolerant to pests and diseases, with good stalk standability and yield stability across environments. Lignin-deficient tracheary elements probably collapse as their cell wall cannot stand the negative pressure generated during long and/or intensive transpiration periods (COCHARD, 2002; COCHARD *et al.*, 2008). Hence, the mechanical resistance of the vascular system has to be preserved in plants bred for higher cell wall degradability, but still tolerant to biotic and abiotic stresses.

Despite the fact that several genes involved in cell wall biosynthesis have been mapped from in silico approaches simultaneously with QTL analyses, only a few can be currently considered as strong candidate genes. Discovery of the relevant candidate genes involved in genetic variation of cell wall degradability will be based on a set of investigations. A lot of bio-analysis researches have first to be considered based on sequences of maize and model species for secondary wall formation (Arabidopsis, aspen, eucalyptus, …). Specific maize and C4 grass genes involved cell wall biosynthesis and assembly should also be searched for by compara-

tive data mining of expressed genes in maize and dicotyledonous plants, as it has been done by MITCHELL *et al.* (2007) for specific cell wall carbohydtare genes of C3 grasses. The search for differentially expressed genes in growing stalk internodes of lines differing for their cell wall traits should be strengthened by similar investigations in each type of tissues after their laser micro-dissection. With the release of the maize genomic sequence, colocalizations between cell wall trait QTL and cell wall related genes can be considered based on genes and QTL physical positions. Finally, functional validation of candidates will be based on transposon tagging and/or RNAi deregulation in maize or Brachypodium, and from association genetics investigations in a panel representative of the maize genetic diversity. Available results strengthen the probable major role of transcription and regulation factors in variation of maize cell degradability and investigations should likely first focus on their discovery and role in tissue patterning, assembly and lignification.

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