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Breeding grasses for capacity to biofuel production or silage feeding value: an updated list of genes involved in maize secondary cell wall biosynthesis and assembly

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Abstract

In the near future, maize, sorghum, or switchgrass stovers and cereal straws will be a significant source of carbohydrates for sustainable biofuel production, in addition to the current use of grass silage in cattle feeding. However, cell wall properties, including the enzymatic degradability of structural polysaccharides in industrial fermenters or animal rumen, is greatly influenced by the embedding of cell wall carbohydrates in lignin matrix, and the linkages between lignins, *p*-hydroxycinnamic acids, and arabinoxylans. Breeding for higher and cheaper biofuel or silage production will thus be based on the discovery of genetic traits involved in each cell wall component biosynthesis and deposition in each lignified tissue. Due to its considerable genetic and genomic backgrounds, maize is the relevant model species for identifying traits underlying cell wall degradability variations in grasses. Maize genes involved or putatively involved in the biosynthesis of cell wall phenolic compounds, cell wall carbohydrates and regulation factors were therefore searched for using data available in grass, *Arabidopsis*, and woody species (mostly poplar and eucalyptus). All maize ortholog genes were searched for using protein sequences and a "blastp" strategy against data available in the www.maizesequence.org database. Genes were also mapped in silico considering their physical position in the same database. Finally, 409 candidate genes putatively involved in secondary cell wall biosynthesis and assembly were shown in the maize genome, out of which 130 were related to phenolic compound biosynthesis, 81 were related to cell wall carbohydrate biosynthesis, and 198 were involved in more or less known regulation mechanisms. Most probable candidate genes involved in regulation and assembly of secondary cell wall belonged to the MYB (45 genes) and NAC (38 genes) families, but also included zinc finger and HDZipIII encoding genes. While genes involved in ferulic acid cross-linkages with other cell wall components were little known, several families putatively involved in (arabino)-xylan chain biosynthesis and in feruloyl transfer were shown, including especially arabinosyl-CoA-acyltransferases, feruloyl-AX β-1,2-xylosyl transferases, and xylan-O-3-arabinosyl transferases. This candidate gene list, which focused on genes and orthologs known to be involved in cell wall component biosynthesis and regulation, cannot be considered as exhaustive. Other genes, whose role in cell wall lignification and deposition have not yet been defined, should very likely be added to the list of candidates required for secondary cell wall assembly. Genes encoding proteins of still unknown function should also be added to the list, as several of the latter are probably involved in lignified tissue biosynthesis and deposition.

Keywords: maize, cell wall, silage, biogas, bio-ethanol, lignin, degradability

Introduction

Concerns over global climate changes, together with a growing worldwide demand for energy and the simultaneous "short term" depletion of fossil stocks, have demonstrated out the crucial need for alternative energy resources. In addition to solar, wind, ocean wave, and nuclear energies, second generation biofuels obtained from fermentation of lignocellulose materials have opened new avenues based either on dedicated crops or recovering a large part of agricultural and woody residues. First generation biofuels based on cereal or oleaginous grains compete with food supplies and have been recently rejected by the European Union commissioner for "Climate Action". Most species considered for biofuel production are grasses, with the use of plant straws of C3 and C4 cereals and sugarcane bagasse, and the whole plant use of maize, sorghum, or switchgrass. Grasses are also the basis of energy nutrition of dairy and meat cattle, with grazing during spring and early autumn and silage maize feeding during the long periods without meadow growth. In France, nearly

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85% of milk is indeed produced by cows fed several months a year on silage maize. However, although green biomass contains almost the same amount of gross energy as do grains per unit of dry matter, the stover energy value is significantly lower, only reaching in wheat straws 33% of maize grain value both in the digestive tracts of animals and in industrial enzymatic fermenters. The biological conversion of cell wall carbohydrates, mainly located in the secondary lignified plant cell walls, into fermentable sugars is hindered by their association with lignins and *p*-hydroxycinnamic acids.

Lignification and the development of a vascular system have allowed plants to leave aquatic habitats and to acquire erect growth. Lignins also impart hydrophobicity to vascular elements allowing water and nutriment transportation. Lignins, with cellulose, contribute to the mechanical properties and structural integrity of tissues, and lignin-deficient tracheary elements may collapse as their cell wall cannot stand the negative pressure generated during long and/or intensive transpiration periods (Cochard, 2002; Cochard et al, 2008). Finally constitutive and neoformed lignins are involved in mechanisms related to disease and pest tolerances. The embedding and cross-linkages between phenolics and carbohydrates thus prevent physical access of enzymes to cell wall carbohydrates and strongly limit their enzymatic hydrolysis. Model studies, in which the degrees of polysaccharide-polysaccharide and lignin-polysaccharide cross-linkages are controlled, established that the former primarily impede the rate of carbohydrate degradation, while the latter impede both the rate and extent of carbohydrate degradation (Ralph, 2010). 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).

Grain maize or “corn” is likely the plant species in which genetic improvements for agronomic traits were the most remarkable during the last century in the USA and over the last six decades in Europe. In forage maize (Barrière et al, 1987; Barrière et al, 2004a; Barrière et al, 2005), the genetic progress in yield was close to 0.17 t ha^{-1} per year for hybrids registered in France between 1986 and 2000 and seemingly has continued up to now (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^{-1} per year (Barrière et al, 1987). However, a significant drift of hybrid cell wall digestibility towards lower values was simultaneously observed during the same period. Based on data from the long term experiment on silage maize feeding value and cell wall digestibility (NDFD, Neutral Detergent Fiber Digestibility) measurements with sheep at INRA Lusignan (Barrière et al, 2004a), hybrids with a lower NDFD than the well-known old early hybrid LG11 (NDFD = 50.2%) were 32, 63, 75, 84, and 93% in each of the registration periods before 1981, 1989, 1994, and 1999, and in or after 1999, respectively. This decline in average cell wall digestibility, which has indeed been mainly observed since 1980, was mostly a consequence of the introduction of Iodent and BSSS genetic resources in early and medium-early line breeding, together with the phasing out of flint lines with high cell wall digestibility but with poor standability and yield. This decline, which was also related to the focus of breeders on (grain) yield and standability, has now ceased with the breeding of specialized silage maize and the use of a digestibility criterion in forage maize registration since 1986 in the Netherlands and since 1998 in France. While the cell wall degradability of the best modern hybrids does not yet equal that of the best older types such as INRA258, several currently registered French hybrids have cell wall digestibility close to that of DEA-type hybrids. However, unlike yield or stress tolerance for which steady improvements have been observed, the energy value of currently released silage maize varieties plateaus. This is due to the fact that cell wall degradability has not been sufficiently taken into account during this era of breeding. Maize cropping for silage use in Europe is therefore now mostly based on varieties bred for whole plant agronomic and quality traits, most of which have medium cell wall degradability. Significant improvements are nevertheless occurring with the registration in the Netherlands of early hybrids such as Aastar, Ayrro, LG30-225, etc, with a cell wall degradability equal or close to 110% of that of the control hybrids.

In any case, maize genetic improvement allowing increased animal or biofuel production based on the non-grain part of plants requires understanding cell wall building rules, as well as genetic and molecular mechanisms involved in secondary wall assembly and cell wall compound biosynthesis, and finally cross-linkage determinants. Gene discovery is thus a major pre-requisite in order to implement effective silage and biofuel breeding programs, based on marker assisted selection (MAS), genome-wide association studies and SNP-based (single nucleotide polymorphism) investigations, genetic resource management, and genetic engineering. Most cell wall (gene) research has been devoted to improving paper pulping conditions towards more environmental friendly processes in woody dicotyledonous and gymnosperms plants (poplar, eucalyptus, pine, spruce, etc). In addition, intensive investigations to decipher cell wall assembly have indeed been based on *Arabidopsis* model plant, and to a lesser extent alfalfa. A wide set of the genes involved in cell wall carbohydrate and phenolic compound biosynthesis, and their regulation, is thus currently available in *Arabidopsis*. However, a list mainly focused on

carbohydrate related genes has also been proposed for maize (Penning et al, 2009). Nevertheless, only limited information is indeed available for maize and grass cell wall gene regulation and lignified tissue patterning in leaves and stems. Firstly, this is due to the fact that the lignin pathway has not really been investigated in the rice model plant, or in the more recent *Brachypodium* or *Setaria* models, and secondly, because, in contrast to dicotyledonous plants, the vascular system of maize and non-woody monocotyledons is characterized by the absence of bifacial cambium and secondary growth. The major role of *p*-hydroxycinnamates in secondary wall structure is also specific to grass plants. However, the emergence and evolution of lignified and vascular tissues was indeed based on a preexisting poly-phenolic pathway (Boyce et al, 2003), followed by millions of years of divergent evolution in grasses and dicotyledons. Many results obtained in plant genetics and genomics of lignification have nevertheless illustrated a significant commonality in cell wall carbohydrate and phenolic compound biosynthesis in all plants. However, even if they have not yet been established, significant differences between species groups could be particularly expected for genomic traits involved in regulation and assembly of lignified tissues. The transcription factors families have disproportionaly expanded, and the regulatory networks diverged, so that the eudicot model is not wholly generalizable to grasses (Handakumbara and Hazel, 2012).

Objective and methodologies

The objective of this investigation was therefore to describe the composition and organization of the maize secondary cell wall, and then to list maize candidate genes possibly involved in the corresponding metabolic pathways. Maize orthologs of transcription and regulation factors described in other species, and mostly dicotyledonous species, were especially considered as candidates. An e-value at least lower than e^{-75} was retained during ortholog searches based on protein sequences. Maize candidate genes were finally put into eight groups including 1) genes involved in monolignol and *p*-hydroxycinnamic acid biosynthesis, and those involved in monolignol polymerization, 2) genes involved in cellulose biosynthesis and cellulose fiber assembly, 3) genes involved in arabinoxylan and related compound assembly, 4) genes involved in feruloylation and acylation of cell wall components, 5) genes of the shikimate pathway, 6) genes of the S-adenosyl-L-methionine cycles, 7) MYB, NAC, and transcription factors involved in cell wall regulation, and 8) miscellaneous genes involved in lignified tissue biosynthesis or assembly. These eight different sets of genes likely are not equally important in determining cell wall degradability variations. Gene GRMZM names and physical positions were based on the maize B73 sequence (www.maizesequence.org, release v2 5b.60). The consensus physical map used to illustrate gene repartition on the maize genome was drawn with markers mapped during the QTL investigations at INRA Lusignan (Méchin et al, 2001; Roussel et al, 2002; Génoplante unpublished data, 2007; Barrière et al, 2008; Riboulet et al, 2008a; Barrière et al, 2012; Courtial et al, 2013). Several markers were however added in areas surrounding centromers based on their physical position given in the MaizeGDB database (all IDP markers), as these locations, in which recombinations are uncommon, were poorly marked.

Phenolic constituents of maize and grass cell walls

The lignified secondary wall of grasses is a composite material with phenolic compounds, cellulose microfibrils, an amorphous matrix consisting predominantly of glucurono-arabinoxylans, and only very few pectins. Phenolic compounds are comprised of lignins and cross-linked *p*-coumaric (pCA) and ferulic (FA) acid derivatives, along with the array of FA dehydrodimer (diFA) derivatives.

Grass lignins result from the combinatorial radical coupling of p-coumaryl, coniferyl, and sinapyl alcohols, giving rise to *p*-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) monomeric units. The average relative frequencies of each H, G, and S unit released by thioacidolysis of native lignins of mature maize internodes were shown to be nearly equal to 3, 37, and 60%, respectively (Lapierre, 1993). A large range of variation in monomer proportions was nevertheless shown between maize lines and the S/G ratio ranged between 1.0 and 1.8 (Méchin et al, 2005). In addition, significant variations in H unit proportions were also shown (Riboulet et al, 2008b). The H, G and S units of grass lignins are interconnected through labile β-O-4 ether bonds representing, depending on lines, from 15 to 25% of between-monomer linkages, and through a series of resistant carbon-carbon and biphenyl ether linkages. While lignins are often referred to as branched three-dimensional structures, lignins are in fact largely linear polymers. 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 made of monomers essentially linked by β-β, β-5, and β-O-4 linkages. The low, but significant amount of H units, which is nearly five times higher than in dicotyledonous plants, impact the properties of the lignin polymer as these units increase the frequency of resistant inter-unit bonds. In addition, the degree to which lignin polymers incorporate various phenolics in place of the three regular constitutive monolignols is likely underappreciated (Ralph, 2010). The incorporation of free FA in lignins through bis-8-O-4 cross-coupling, which occurs at very low, but significant, levels in normal maize plants but may build up in CCR- and CAD-deficient plants,

provides a third branching point in lignin polymers (Ralph et al, 2008b; Barrière et al, 2013). In addition to FA and hydroxycinnamaldehydes, unusual monomers, including acylated hydroxycinnamyl alcohols, dihydro-hydroxycinnamyl alcohols, hydroxybenzaldehydes and other hydroxycinnamic acids, can be incorporated in lignins of wild-type plants (Vanholme et al, 2012). Moreover, currently obtained results seemingly show that plants could tolerate shifts in lignin composition with no or lower impact on growth than observed for reduced lignin contents (Eudes et al, 2012; Vanholme et al, 2012). The fact that plants can readily incorporate alternative unusual monomers with modified physicochemical and stereochemical properties could be a basis for original plant improvement for both animal feeding and biofuel production.

The participation of *p*-hydroxycinnamates in cell wall composition and organization is a specific trait of grass plant 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, even if 10% of *p*-coumarate can be found on maize G units (Grabber et al, 1996; Lu and Ralph, 1999). Most *p*-coumarate accretion occurs in tandem with lignification and *p*-coumarate accumulation is thus a relevant indicator of lignin deposition. In maize, S unit acylation occurs at the monolignol level, and from 25 to 50% of S lignin units may thus be acylated by pCA (Lu and Ralph, 1999; Morreel et al, 2004; Grabber and Lu, 2007; Martinez et al, 2008; Ralph et al, 2008a). Acylation has a marked influence on the bonding mode of S lignin units, on the spatial organization of lignins and consequently on their capacity to interact with polysaccharides. During monolignol polymerization, sinapyl alcohol is only slowly oxidized by maize peroxidases. Conversely, an oxidation shuttle operates in acylated conditions because the pCA component of the S-pCA conjugate is readily oxidized, with the subsequent transfer of its oxidation state to sinapyl alcohol (Boudet, 2000; Ralph et al, 2004; Hatfield et al, 2008).

Even if ferulate is at first the major *p*-hydroxycinnamic derivative in young grass cell walls, at least 50 to 70% of the alkali-labile ferulate deposition occurs during secondary wall lignification (Iiyama et al, 1990; Morrison et al, 1998; MacAdam and Grabber, 2002; Grabber et al, 2004). Ferulic units are primarily esterified to glucurono-arabinoxylans, and lignins and arabinoxylans are secondarily bridged through FA ether-linkages at the β-position of G units. Ferulates thus provide points of growth for the lignin polymer, acts as lignin nucleation sites, and direct cell wall cross-linking (Ralph et al, 1992; Jacquet et al, 1995; Ralph et al, 1995; Ralph, 2010). 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).

Large variations in phenolic component contents have been shown between maize inbred lines or between hybrids. The content in ADL/NDF nearly doubled between 4 and 8% in maize lines having extreme values [NDF and ADL are neutral detergent fiber and acid detergent lignin according to Goering and van Soest (1970), respectively]. A similar two-fold variation was also observed for pCA content, which could be partly related to the variation in lignin contents. Despite their (relatively) low amounts, significant variations have been shown for esterified and etherified FA (esterFA and etherFA) releases after alkaline hydrolysis, as was also highlighted for 8-O-4 and 5-5 diferulates. Finally, variation for the recovery yield of each H, G or S lignin-derived monomer after nitrobenzene oxidation was similarly of a two-fold range between maize lines, with significant consequences on the polymer arrangement and spatial organization.

As a consequence of variable phenolic component contents and organizations, large genetic variations in the *in vivo* or *in vitro* cell wall digestibility of maize plants have been shown, with small genotype x environment interaction effects compared 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% in a set of 26 lines (Barrière et al, 2009) 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)].

Lignin content is the first trait that has been related to cell wall degradability, but breeding for a much reduced lignin content has too many negative consequences on other agronomic qualities. Moreover, variations in lignin content are not the only determinants explaining variations in cell wall degradability. This fact was especially highlighted after correlation and QTL analyses (Barrière et al, 2008; Riboulet et al, 2008b; Zhang et al, 2011a; Barrière et al, 2012), and from cell wall model studies (Grabber et al, 1998; Grabber et al, 2005). Variable colocalizations between cell wall degradability QTLs and phenolic compound QTLs were indeed shown, with several cell wall degradability QTLs that did not colocalize with lignin QTLs. Corroborating negative correlations between cell wall degradability and etherFA releases, and QTL colocalizations (Casler and Jung, 1999; Méchin et al, 2001; Lam et al, 2003; Riboulet et al, 2008b; Jung and Phillips, 2010; Taboada et al, 2010; Jung et al, 2011; Barros-Rios, 2012), the role of ferulate cross-linkages 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 has thus improved cell wall degradability in perennial grasses (Casler and Jung, 1999; Casler et al, 2008). Similarly, breeding for reduced diferulate contents in maize pith increased cell wall polysaccharide degradability (Barros-Rios et al, 2012). Moreover, ferulate cross-linkages were shown to be involved in stalk stiffness (Grabber et al, 1995; Grabber et al, 2000; MacAdam and Grabber, 2002). While nearly one half of intake variations in cows were explained by cell wall degradability (Barrière et al, 2003), scattered but convergent results support the hypothesis that the rest of the genetic variations in intake are related to plant tissue friability and susceptibility to crushing. Consequently, ferulate cross-linkages likely impede silage maize intake as they decrease plant friability (Ciba-Semences, 1990, 1995; Barrière et al, 1995; Jung and Allen, 1995; Barrière et al, 2004b; Fernandez et al, 2004). Correlations and QTL colocalizations pointed out a negative effect of pCA content on cell wall degradability (Barrière et al, 2008; Riboulet et al, 2008b; Zhang et al, 2011a; Barrière et al, 2012). In addition to being a probable direct effect of S unit acylation on lignin polymer geometry, pCA content is likely also a relevant indicator of intensity and length of secondary tissue lignification. Finally, attempts to understand the impact of lignin structure, commonly described by ratios between H, G, and S units, on the susceptibility of the cell wall to enzymatic hydrolysis have led to conflicting results (Méchin et al, 2000; Grabber et al, 1997; Grabber et al, 2009). However, based on correlations and QTL colocalizations, increased proportions of H units and S units likely contribute, for different reasons, to lowering of cell wall degradability (Riboulet et al, 2008b). As was considered for pCA contents, a higher proportion of S units in lignins might indicate a higher proportion of mature secondary wall in tissues. Lignin structure can also be characterized by the yield of monomers released after thioacidolysis, and a greater proportion of β-O-4 linkage in the lignin polymer has been shown to be negatively correlated with cell wall degradability (Zhang et al, 2011a). This latter fact could be explained by the more extended shape of β-O-4 lignins, maximizing the masking effect on carbohydrate polymers, in comparison to the more globular shape of condensed 5-5 and β-5 lignins (Besombes and Mazeau, 2005).

Genes involved in the upstream parts of cell wall carbohydrate biosynthesis

Cellulose and glucurono-arabinoxylans are the main constituents of lignified secondary walls. 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). All these genes, which are indeed key components in multiple plant metabolisms, were not *a priori* considered as putative candidates involved in variation of cell wall degradability. However, a member of one of these multigene families might be specifically involved in a metabolon devoted to cellulose or arabinoxylan biosynthesis, and therefore could be involved in cell wall variation with consequences on biofuel production capabilities. A nucleotide sugar transporter (OsNST1 or Os02g40030) was thus shown underlying the *brittle-culm-14* (*bc14*) mutation in rice (Song et al, 2011; Zhang et al, 2011b). Mutant *bc14* plants have reduced cellulose content, irregular orientation of cellulose microfibrils, and higher xylan extractability. This set of traits improves the extractability of all cell wall components. Only two close orthologs were shown in maize (Supplementary Table 1).

Genes involved in cellulose biosynthesis and cellulose fiber organization

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. Twelve *CesA* genes have been described in maize (Appenzeller et al, 2004), and further shown in the maize sequence database. Deficiency in one or another *CesA* gene impedes cellulose biosynthesis and modifies the orientation or organization of cellulose microfibrils, with consequences on 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; Taylor et al, 2000). In addition to *CesA* genes, 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 appar-

ent alteration in cell wall composition, and the rice *brittle culm12* mutant are altered in kinesin proteins (Zhong et al, 2002a, Zhang et al, 2010). Kinesins are ATP-driven microtubule-based motor proteins with diverse functions in plant growth and developmental processes. These functions include the mediation of cortical microtubule activity and the orientation of cellulose microfibrils during differentiation of xylem cells (Zhong et al, 2002a). 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* both have orthologous genes in maize, whose involvement in cellulose deposition is still unknown. Rice *brittle culm1* and maize *brittle stalk2* mutants, which have reduced mechanical strengths, are affected in orthologs of COBRA-like proteins encoding putative glycosylphosphatidylinositol-anchored proteins (Li et al, 2003a; Ching et al, 2006; Sindhu et al, 2007; Dai et al, 2011). These COBRA-like proteins were considered to be involved in a patterning of lignin-cellulose 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). Moreover, it was considered that KORRIGAN activity facilitates cellulose biosynthesis in a way that increases the amount of non-crystalline cellulose (Takahashi et al, 2009), which is the preferentially hydrolysed part of cell wall cellulose. Finally, two chitinase-like proteins CTL1 (At1g05850) and CTL2 (At3g16920) have been shown to be involved in regulation and biosynthesis of cell wall carbohydrates (Zhong et al, 2002b; Hossain et al, 2011). Mutations in both *CLT1* and *CLT2* genes induced ectopic deposition of lignin (*CTL1* also named *ELP1* for *Ectopic Deposition of Lignin in Pith 1*, and *POM1*). The *CTL2* gene was shown predominantly expressed in stems. These two CTL genes encode proteins which have no chitinase activity, and have the same unique ortholog in the maize genome, located in bin 7.03. Associations of cellulose with hemicelluloses are important for microfibril spacing and for maintaining cell wall tensile strength. The two latter chitinase-like proteins were considered to play a key role in establishing interactions between cellulose microfibrils and hemicelluloses (Sánchez-Rodríguez et al, 2012). In fact, many components are associated with the CesA complexes, some of which are specific to lignified secondary wall assembly (Endler and Persson, 2011). While several of these gene functions can induce differences in mechanical stem stiffness, it is still unclear whether such variations can induce differences in intrinsic cell wall degradability. However, variation in mechanical tissue quality intake have been considered to be involved in the duration of chewing and silage intake for dairy cows (Barrière et al, 2004b; Fernandez et al, 2004; Barrière et al, 2009). A lower mechanical reistance of tissue would also reduce the cost of biomass crushing in industrial processes. As a whole, 23 maize genes were shown with probable or putative function in cellulose biosynthesis, deposition, and fiber organization (Supplementary Table 1).

Genes involved in arabinoxylan biosynthesis

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 (G6DH). UDP-D-GlcA is next converted into xylose in a reaction catalyzed by UDP-D-GlcA decarboxylase. 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), which showed a 50% reduction in L-arabinose in leaf cell walls. The UDP-L-arabinose produced by the UDP-D-xylose-4-epimerase is in the pyran form and requires a UDP-arabinopyranose mutase (UAM) to be converted to UDP-L-arabinofuranose, which is the form transferred to the xylan backbone (Konishi et al, 2007; Konishi et al, 2010). UAM were shown to be encoded by genes of the reversibly glycosylated polypeptide/glycosyltransferase 75 family. RNA interference lines in rice targeting members of this family showed a decreased content in arabinofuranose in their wall (up to 44%) and a reduced level of xylan substitution (Konishi et al, 2011). In addition, the involvement of UDP-sugars in the biosynthesis pathway of hemicellulose polysaccharides could strengthen the possible and simultaneous involvement of UDP-arabinose in feruloylated arabinoxylan formation (Buanafina, 2009).

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). Furthermore, co-expression of *Arabidopsis* IRX9 and IRX14 in tobacco BY2 cells lacking xylan resulted in an increase in xylosyltransferase activity onto a Xyl4 acceptor (Lee et al, 2012). Several genes of the glycosyltransferase GT43 family were thus shown to be more specifically expressed in grasses (Mitchell et al, 2007), corresponding to plausible maize candidate genes encoding xylan xylosyltransferases. In addition, the *IRX10* mutants of *Arabidopsis* have similar characteristics to those of the IRX9 mutant, suggesting that IRX10-like glycosyltransferases (GT47 family) could also play a role in the elongation of the xylan backbone (Brown et al, 2009; Wu et al, 2009; Oikawa et al, 2010). Mutants in the rice IRX10 orthologous gene were recently reported not to be affected in the length of their xylan polymer, despite a reduction both in the xylose and the xylan contents (Chen et al, 2012a). A direct role of IRX10 in xylan elongation seems therefore unlikely. While no changes were simultaneously observed in lignin content, the rice *OsIRX10* mutant also displayed a higher cell wall saccharification efficiency (Chen et al, 2012a). IRX10-like genes may have indeed a different function in grasses than in *Arabidopsis*. Members of the GT47 family were proposed to carry out in grasses both xylan α-1,2- and/or α-1,3-arabinosyl transferase activities, allowing the transfer of an arabinosyl residue onto an X(X) chain (Mitchell et al, 2007). Nevertheless, the involvement of members of the GT47 family in the transfer of arabinose onto xylan is still only hypothetical. In addition, several members of the GT61 family (clade A) were shown to be xylan O-3 arabinosyltransferases in wheat and rice (Anders et al, 2012). Based on RNA interference, the deregulation of one GT61 gene induced a decrease in α-1,3 linked arabinose, but also in total arabinoxylans. The latter fact suggested a substitution requirement for continued backbone synthesis. Other members of the GT61 family were found in rice to be β-(1,2) xylosyltransferase acting on arabinosyl residues linked to xylans on C3 (Chiniquy et al, 2012), with orthologs in maize. Members of the GT8 glycosyltransferase protein family are also involved in the biosynthesis of secondary cell wall xylans. The *Arabidopsis IRX8* mutant has thus a 60% reduction in xylan content (Persson et al, 2007). In poplar, two GT8 members were shown abundantly and specifically expressed in the differentiating xylem. RNAi down-regulated lines for both GT8 glycosyltransferase genes had a nearly 33% reduction in stem wood xylan content, no change in cellulose quantity, and an increase in lignin content ranging between 10 and 25%. These transgenic plants exhibit thinner fiber cell walls in stem xylem, a brittle wood phenotype, and reduced stem modulus of rupture (Li et al, 2011a). Xylan and carbohydrate content and organization in the secondary cell wall are thus likely, with ferulate cross-linkages, more important factors than lignin content affecting the stiffness and fracture strength of tissue, with very probable consequences on forage intake by cattle.

Arabinoxylans in maize (and grasses) are in fact glucurono-arabinoxylans, with xylan substitution by arabinose on the C2 and/or C3 position, and by (4-O-methyl-) glucuronosyl on C2. Candidate genes for (4-O-methyl-) glucuronic transfer can be found in the GT8 family since xylans of *Arabidopsis* double mutants in the *GT8*/*GUX* genes were shown to be devoid of glucuronosyl substitution (Mortimer et al, 2010). Three orthologs of the *Arabidopsis* genes *GUX1* (At3g18660), *GUX2* (At4g33330), and *GUX3* (At1g77130), which encode proteins located in the Golgi apparatus, with a glucuronosyltransferase activity, were shown in the maize genome. The acetylation of xylan backbones at C-2 and/or C-3 positions could affect about 40% of xylosyl residues (Ebringerova et al, 2005; Lee et al, 2011a; Manabe et al, 2011; Gille and Pauly, 2012; Saulnier et al, 2012). The biological significance of polysaccharide O-acetylation is not fully known, but acetylation was shown to affect the physicochemical properties of cell wall xylans. Moreover, the presence of acetyl esters negatively impedes biomass enzymatic saccharification, and the release of acetate and conversion products of acetate also are inhibitory to the micro-organisms used during cell wall sugar fermentation into ethanol (Manabe et al, 2011; Gille and Pauly, 2012). Genes catalysing the O-acetylation of xylan have long been unknown in plants, while it has been shown that the CAS1 gene from the yeast *Cryptococcus neoformans* was involved in the O-acetylation of its main capsular polysaccharide (Janbon et al, 2001). Four orthologs of the Cap1s encoded protein were recently shown in *Arabidopsis*, which are regulated by the NAC factor SND1 (Lee et al, 2011a). These four REDUCED WALL ACETYLATION genes (*RWA1-4*) have close sequence similarities. *RWA1*, *RWA3* and *RWA4* genes were shown to be expressed in both xylem cells and interfascicular fibers, while *RWA2* was only expressed in xylem cells. *RWA2* mutant plants had an overall acetylation reduction of 20% in wall polymers including xylans (Manabe et al, 2011). A second family of protein involved in plant polysaccharide O-acetylation was identified, based on investigations with the *AXY4* mutants of *Arabidopsis* which lacked O-acetyl-substituents on xyloglucan chains (Gille et al, 2011). The *AXY4* (*TBL27*) and *AXY4*-like (*TBL22*) genes belong to the trichome birefringence-like (TBL) family (Bischoff et al, 2010a), which includes 46 members in *Arabidopsis*. Other members of the TBL family were proposed to encode additional wall polysaccharide specific O-acetyltransferases (Gille et al, 2011; Gille and Pauly, 2012). However, the latter fact has not yet been established, and similarly the possible role of maize orthologs in xylan O-acetylation is still hypothetical.

Taking into consideration the genes involved in xylose, arabinose, and arabinoxylan chain biosyn-

thesis and acetylation, 57 genes were shown in the maize genome, most of which had transferase activities (Supplementary Table 2).

Genes involved in the shikimate pathway, upstream the monolignol pathway

In plants, the shikimate pathway links the carbohydrate metabolism to the biosynthesis of aromatic amino acids (phenylalanine, tyrosine, and tryptophan) and, consequently, to the phenylpropanoid pathway. In the first step, phosphoenolpyruvate and erythrose-4-phosphate react to form 3-deoxy-D-arabinoheptulosonate-7-phosphate (DAHP), in a reaction catalyzed by the DAHP synthase. DAHP is transformed to 3-dehydroquinate (DHQ), in a reaction catalyzed by the DHQ synthase. DHQ is dehydrated to 3-dehydroshikimic acid by the dehydroquinase, which is finally reduced to shikimic acid by the shikimate dehydrogenase. The next enzyme involved is the shikimate kinase, which catalyzes the ATP-dependent phosphorylation of shikimate into shikimate 3-phosphate. The shikimate 3-phosphate is coupled with phosphoenol pyruvate to give the 5-enolpyruvylshikimate-3-phosphate in a reaction catalyzed by the 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase. Then, the 5-enolpyruvylshikimate-3-phosphate is transformed into chorismate by a chorismate synthase, and chorismate gives rise to prephenate and arogenate in two reactions, the order of which has not yet been established, catalyzed by the chorismate mutase and the prephenate aminotransferase, respectively, using glutamate as the nitrogen source. Phenylalanine and tyrosine are finally produced from arogenate in reactions catalyzed by the arogenate/prephenate dehydratase (ADT/PDT) and arogenate/prephenate dehydrogenase (ADH/PDH), respectively (Maeda and Dudareva, 2012; Vanholme et al, 2012). Genes encoding enzymes of this pathway have been described in a more or less reduced number of plant species, allowing the search for orthologs in maize. The carbon flow in the shikimate pathway, the regulations of the shikimate pathway genes, and the phenylalanine (and tyrosine) supply to PAL (and TAL) enzymes, may thus directly affect biosynthesis of phenylpropanoid compounds, including monolignols. For the shikimate pathway, 28 genes were found in the maize genome, but all the members of each multigene family are probably not involved in aromatic amino acid supply to the monolignol pathway (Supplementary Table 3).

Genes involved in the monolignol biosynthesis pathway

The first step of monolignol biosynthesis, which occurred downstream the shikimate pathway, is the deamination of L-phenylalanine into cinnamic acid. Successive steps including hydroxylation and methylation on the aromatic ring further lead to the production of the three monolignols which are then exported to the cell wall and polymerized into lignins. Most genes involved in monolignol biosynthesis belong to small multigene families, with different members possibly involved in different metabolons corresponding to each monolignol biosynthesis and/or to their biosynthesis in each type of lignified tissue. However, not all family members are likely involved in constitutive lignin biosynthesis, and some members probably correspond to biotic or abiotic stress-response lignins. Some members of the upstream part of the pathway could also be specifically involved in the biosynthesis of other phenolic compounds such as suberins or flavonoids. Moreover, lignin pathway enzymes are likely organized as endoplasmic-reticulum-associated multi-enzyme complexes (Winkel, 2004). The probable different enzymatic complexes should 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 regulation mechanisms.

The deamination of L-phenylalanine into cinnamic acid is catalyzed by a phenylalanine ammonia lyase (PAL) enzyme. Maize PALs also have a tyrosine ammonia lyase (TAL) activity (Higuchi et al, 1967; Roesler et al, 1997), catalyzing tyrosine deamination into pCA. Ten PAL genes are present in the maize genome, corresponding to three groups of probably duplicated genes, plus an isolated gene which has a more distant sequence. 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), possibly highlighting a major role of this member in the family. The hydroxylation of cinnamic acid is then catalyzed by a cinnamate 4-hydroxylase (C4H, two genes) and the resulting *p*-coumaric acid is then converted into coumaroyl-CoA by a 4-CoA ligase (4CL, two genes). In *Arabidopsis*, the down-regulation of the *4CL1* gene reduced the G unit content in lignins, but did not affect the S unit content (Lee et al, 1997). This fact strengthened the existence of metabolons with the preferential or specific involvement of different members of each multigene family in the biosynthesis of each monolignol and/or their biosynthesis in given tissues. The conversion of coumaroyl-CoA into caffeoyl-CoA through the formation of shikimate (or quinate) esters involves a hydroxycinnamoyl-CoA shikimate/quinate hydroxycinnamoyl transferase (HCT, two genes) also having a reverse action, and a p-coumaroyl-shikimate/quinate 3-hydroxylase (C3H, two genes) grafting the hydroxyl residue on the aromatic ring (Schöch et al, 2001; Hoffmann et al, 2003; Hoffmann et al, 2004; Mahesh et al, 2007; Shadle et al, 2007). An alternative route towards the 3-hydroxylation of the aromatic ring has been shown in poplar. The heterodimeric C4H/C3H protein complex catalyzes the conversion of *p*-coumaric acid into caf-

feic acid (Chen et al, 2011), allowing the production of caffeoyl-CoA in a reaction catalyzed by a 4CL. The conversion of caffeoyl-CoA into feruloyl-CoA is then catalyzed by caffeoyl-CoA O-methyltransferases (CCoAOMT, five genes), but CCoAOMT4, which is in duplicate position with CCoAOMT5, appeared to be of little importance in constitutive lignification (Guillaumie et al, 2007a; Guillaumie et al, 2007b; Riboulet et al, 2009). Caffeoyl-CoA and feruloyl-CoA are two hub-compounds, towards the synthesis of coniferyl and sinapyl alcohol, and also ferulate derivatives.

Conversions of activated *p*-coumaroyl-, caffeoyl-, and feruloyl-CoA compounds into aldehydes are mainly driven by the ZmCCR1 cinnamoyl-CoA reductase, even if a *ZmCCR2* gene is present in the maize genome. Similarly, the reduction of *p*-hydroxy-cinnamaldehydes into alcohols is also mainly catalyzed by the ZmCAD2 cinnamyl alcohol dehydrogenase, while the role of ZmCAD1 is not really understood. These two types of CAD genes and proteins 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). An EgCAD1-type CAD activity has been described in maize by Kanazawa et al (1999), while an EgCAD1-type enzyme was proven to be involved in the synthesis of coniferyl alcohol in tobacco cell wall (Damiani et al, 2005). The major roles of both ZmCCR1 and ZmCAD2 in the two last steps of monolignol biosynthesis are highlighted by the effects of corresponding mutants. *ZmCAD2* mutations have been associated with the maize brown-midrib bm1 phenotype, inducing higher cell wall degradability, lower lignin content, an incorporation of aldehydes in the lignin polymer, and no change in the syringyl/guaiacyl (S/G) ratio (Halpin et al, 1998; Barrière et al, 2004c; Chen et al, 2012b; Barrière et al, 2013). Maize CAD down-regulated RNAi plants, which did not presented the brown-midrib phenotype, nor changes in stem lignin content, were however shown to be more degradable, with an improved cellulosic bioethanol production (Fornalé et al, 2012). Even if similarly no brown-midrib phenotype was shown, a transposon-tagging mutation in the *ZmCCR1* gene also induced reduced lignin content and higher cell wall degradability. In addition, H units were released in lower amounts from the *ZmCCR1* mutant plants compared with the normal ones, with simultaneously an increase in the S/G ratio in mutants (Tamasloukht et al, 2011). Finally, other members of the CCR and CAD families could correspond to genes mainly involved in defense processes, which are likely able to partially compensate the impaired enzymatic activities in CCR1 and CAD2 mutant plants.

Ferulate 5-hydroxylase (F5H) catalyzes the 5-hydroxylation of coniferaldehyde (and to a lesser extent, coniferyl alcohol) into 5-hydroxyconiferaldehyde (5-hydroxyconiferyl alcohol, respectively). Two *F5H* genes are present in maize genome, *F5H1* with a strong expression in maize stalks and *F5H2* mostly expressed in roots (Guillaumie et al, 2007a; Riboulet et al, 2009). 5-Hydroxyconiferaldehyde is methylated into sinapaldehyde by the caffeic acid O-methyltransferase (COMT), which is the only gene of the maize monolignol pathway that does not belong in maize to a small multigene family. The COMT enzyme has a much greater affinity for the 5-hydroxyconiferyl aldehyde than for the alcohol (Li et al, 2000; Parvathi et al, 2001; Louie et al, 2010), and despite its denomination, the COMT enzyme has no *in vivo* activity on caffeic acid (Davin et al, 2008). The importance of the maize COMT gene in lignin biosynthesis was previously established from mutant or transformed plant investigations, and from association studies. The brown-midrib *bm3* mutation, which occurred in the *COMT* gene, first induced a reduced COMT activity and reduced lignin content (Grand et al, 1985; Vignols et al, 1995; Guillaumie et al, 2008). Plants with the *bm3* mutation also had greatly improved cell wall degradability, and reduced S/G ratio with a reduction to 40 % of S units released after thioacidolysis with significant incorporation of 5-hydroxy-coniferaldehyde (Kuc and Nelson, 1964; Barrière et al, 2004c). Similar results were shown in COMT down-regulated plants (Piquemal et al, 2002; He et al, 2003; Pichon et al, 2006).

Biosyntheses of coniferyl and sinapyl alcohols are possibly based on two different preferential routes starting from caffeoyl-CoA, at least in several plant species (Guo et al, 2001; Parvathi et al, 2001; Lee et al, 2011b). Coniferyl alcohol likely mostly originates from a synthesis of coniferaldehyde after a methoxylation of caffeoyl-CoA into feruloyl-CoA. Even if, depending on the species, a variable part of syringyl alcohol could also derive from the same pathway, syringyl alcohol could be produced from a first CCR-catalyzed reduction of caffeoyl-CoA into caffeoyl aldehyde, and then a methoxylation on this last compound. This methoxylation of caffeoyl aldehyde into coniferaldehyde has been considered to be catalyzed by the caffeic acid O-methyltransferase (COMT) in several studies on dicotyledonous plants (Li et al, 1997; Guo et al, 2001; Parvathi et al, 2001; Chen et al, 2006; Do et al, 2007; Lee et al, 2011b; Zhao and Dixon, 2011; Gray et al, 2012). The route duality and the involvement of COMT in a methoxylation step other than on 5-hydroxyconiferaldehyde have not been established in grasses. However, since the disruption of the COMT gene in maize *bm3* mutants did not completely prevent the synthesis of syringyl alcohol, an alternative methoxylation pathway should exist in maize. On the contrary, in the *Arabidopsis AtOMT1* mutant, the lignin content in S units is reduced to a value close to zero (Goujon et al, 2003). Because CCoAOMT en-

zymes have a strict affinity for CoA-esters (Martz et al, 1998; Meng and Campbell, 1998; Parvathi et al, 2001), they cannot be considered as candidates involved in 5-hydroxy-coniferaldehyde methoxylation. Conversely, several ZRP4-like OMT are expressed in lignifying tissue of maize stems (Guillaumie et al, 2007a,b). Consequently, their role is very likely not limited to methylation of suberin sub-unit precursors in plant roots as initially described (Held et al, 1993). At least one ZRP4-like OMT could thus contribute to methoxylate the C5 position of the phenolic ring during monolignol biosynthesis in maize. In agreement with this hypothesis, the expression of two ZRP4-like OMT was increased by nearly two fold in *bm3* young and silking plants (Guillaumie et al, 2007a; Guillaumie et al, 2008).

Genes involved in monolignol transport and polymerization

After their biosynthesis, the three monolignols are sequestred into vacuoles as 4-O-glucosides, while monolignol aglycones are transported across membranes (Lim et al, 2005; Escamilla-Trevino et al, 2006; Miao and Liu, 2010; Alejandro et al, 2012; Liu, 2012; Vanholme et al, 2012). Maize genes were searched for as the orthologs of *Arabidopsis* uridine-diphosphate-glucosyltransferases (UGT), involved in glucosylation of coniferyl and sinapyl alcohols and strongly expressed in lignifying tissues (Lim et al, 2005; Lanot et al, 2006). Complementarily, orthologs of pine and *Arabidopsis* β-glucosidases (β-Glu45 and β-Glu46), which encode proteins involved in the release of monolignol aglycone from its glucosidic form at the cell wall with narrow specificity towards the three monolignol glucosides (Dharmawardhana et al, 1995; Escamilla-Trevino et al, 2006), were considered as involved in the corresponding maize monolignol metabolism. ABC transporters are involved in the transport of monolignols across membranes (Sanchez-Fernandez et al, 2001; Samuels et al, 2002; Ehlting et al, 2005; Miao and Liu, 2010; Kaneda et al, 2011; Liu, 2012), a fact that was recently supported by the identification of *AtABCG29* as a gene encoding for an ABC *p*-coumaryl alcohol transporter in rice (Alejandro et al, 2012). The AtABCG29 protein appeared with a great specificity to *p*-coumaryl alcohol, but its mutation nevertheless induced significant and mostly unexplained modifications in all lignin constituents. Other (ABC) monolignol transporters have thus to be evidenced, such as orthologs of eucalyptus ABC transporters expressed in xylem tissues (Rengel et al, 2009). In addition, out of the two maize mostly expressed ABC transporters in plantlets (Guillaumie et al, 2007a), one was significantly under-expressed in *bm2* plantlets, and could therefore be supposed to be preferentially involved in coniferyl alcohol transport (Guillaumie et al, 2007b).

Condensation of monolignols into the lignin polymer occurs via combinatorial radical-radical coupling reactions (Freudenberg, 1959; Ralph et al, 2008a; Vanholme et al, 2012), despite the fact that it has also been considered to occur through an ordered radical coupling driven by dirigent proteins (Davin and Lewis, 2000; Davin et al, 2008). According to Vanholme et al (2012), and considering that radical coupling is a chemically driven process, independent of control by any protein, "any phenolic molecule entering the cell wall region and having the proper chemical kinetic, thermodynamic radical-generation, and cross-coupling propencies can couple into the lignin polymer". This fact helps explain the diversity of monomers that has been currently seen in the lignin macromolecules. In any case, the coupling modes leading to the structure and geometry of lignin polymers are primarily influenced by the polysaccharidic matrix in which tissue 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.

While class III peroxidases have long been considered as the unique class of oxidases involved in lignin polymerization, EST sequencing and expression studies based on lignifying tissues, and mutant investigations, have shown that both laccases and peroxidases are involved in cell wall lignification (Boudet, 2000; Nielsen et al, 2001; Boerjan et al, 2003; McCaig et al, 2005; Cai et al, 2006; Sasaki et al, 2006; Sato and Whetten, 2006; Tokunaga et al, 2009; Fagerstedt et al, 2010; Berthet et al, 2011). Class III peroxidases and laccases belong to multigene families, and consequently, redundancy in their activity has often been suspected. However, the importance of oxidase redundancy is greatly reduced by the fact that many peroxidases or laccases have specific spatio-temporal expression patterns. When considering genes expressed in maize vascular and lignifying tissues, and orthologs of *Arabidopsis* genes expressed in lignifying stems (de Obeso et al, 2003; Bakalovic et al, 2006; Caparros-Ruiz et al, 2006; Guillaumie et al, 2007a; Andersen et al, 2009; Barrière et al, 2009; Riboulet et al, 2009), only five peroxidase and fourteen laccase genes were currently considered in the maize genome. Whether all the latter genes are effectively involved in constitutive lignification, and whether some other members are still unidentified, especially for peroxidases, is not known. However, several investigations suggested that only a few members would be involved in secondary wall assembly. This fact is more likely a consequence of a regulated spatio-temporal expression of peroxidase and laccase genes, rather than a specificity of several family members towards monolignols. 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).

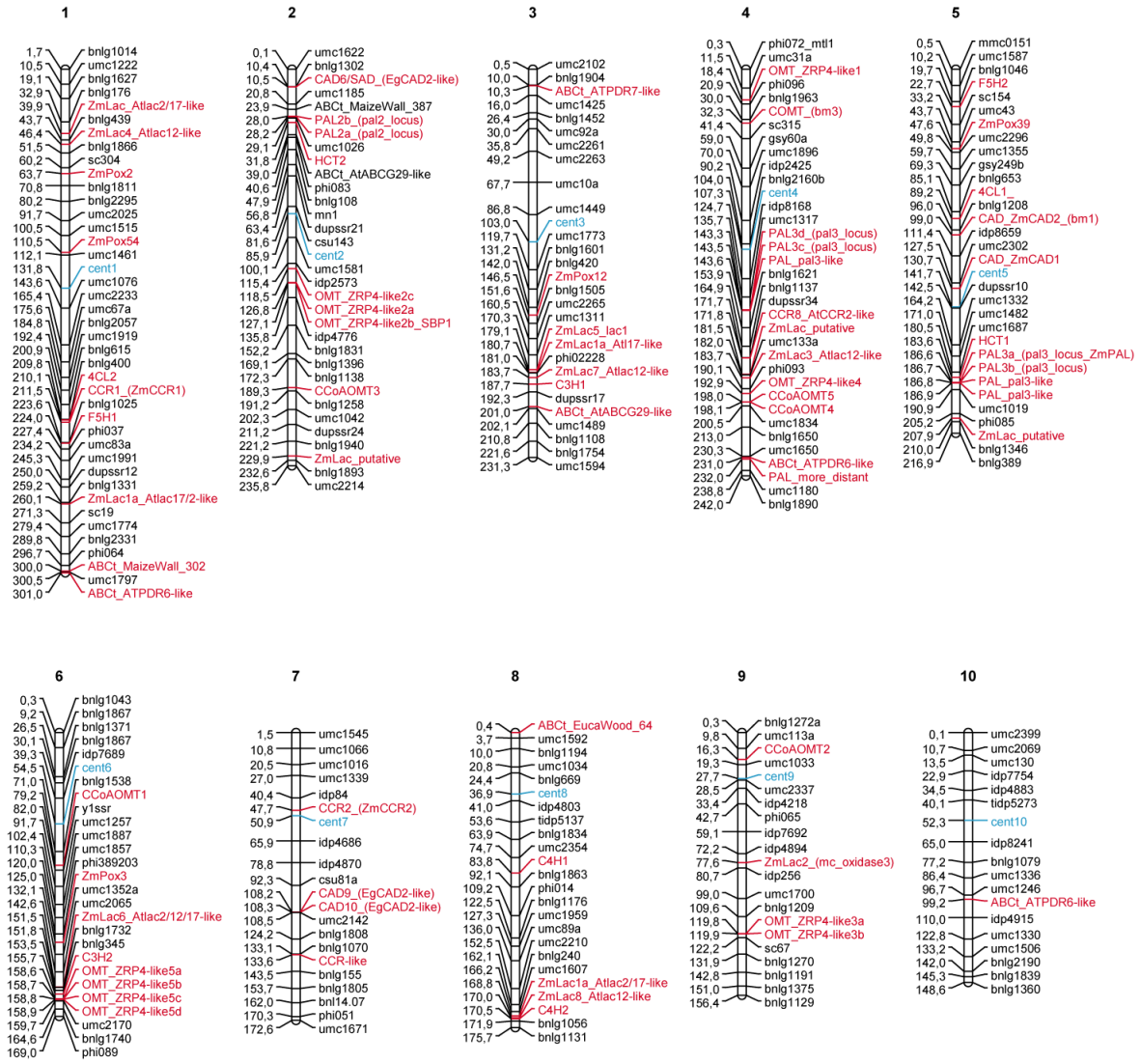

Figure 1 - Physical map of maize genes related to monolignol biosynthesis, transport, and polymerization.

Down-regulation of the tobacco peroxidase TP60 led to plants with lignin reductions of 20 and up to 40-50% of control plants. In the down-regulated line with the most robust changes in lignin content through several generations, plants have thin cell walls and limited secondary wall thickening with an abnormal S2 layer (Blee et al, 2003; Kavousi et al, 2010). Similar results were shown in aspen after deregulation of the PrxA3a peroxidase (Li et al, 2003b). In addition, a MITE insertion disrupting the *ZmPox3* gene was associated with higher cell wall degradability in a set of related maize European flint lines (Guillet-Claude et al, 2004), whereas *ZmPox2* was considered to be involved to a greater extent than *ZmPox3* in maize vascular vessels and epidermis lignification (de Obeso et al, 2003). While the lignin content was not changed, the down-regulation of the poplar *Lac3* laccase gene induced an important alteration of xylem fiber cell walls, with an increase in soluble phenolic compounds (Ranocha et al, 2002). In *Arabidopsis*, laccase *AtLac4* and *AtLac17* double mutants had lignin content that were 35% lower than in control plants, with higher saccharification yields, while the reduction was nearly 13% in single mutants (Berthet et al, 2011). Over-expression of a cotton laccase in poplar plants induced an increase in lignin content in all tested transgenic lines in varying degrees, but as high as 21.5% (Wang et al, 2008). Observed effects on lignin content in (double) peroxidase or laccase mutants or transformants were thus of the same order of magnitude as those observed with monolignol genes, such as in the maize *bm3*/*COMT* mutant, and even higher than in the maize *bm1*/*CAD* mutant.

Considering the different steps involved in monolignol biosynthesis, transport, and polymerization (Figure 1 and Supplementary Table 4), 74 genes were

shown in the maize genome. The genes corresponding to the different enzymatic activities are scattered throughout the whole genome, while conversely clusters of paralogs, which likely correspond to gene duplications, were shown for PAL, ZRP4-like OMT, and laccase genes.

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; Ranocha et al, 2001; Moffatt et al, 2002). The methylenetetrahydrofolate reductase (MTHFR) catalyzes the conversion of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate (5-methylTHF), a cosubstrate for homocysteine remethylation to methionine (Goyette et al, 1994). The methionine synthase (MS) then catalyzes the synthesis of methionine from homocysteine with the supply of the methyl radical from 5-methylTHF conversion into THF. 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). The 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). Up until recently, no data had shown that methyl group availability could be a limiting factor in monolignol biosynthesis. However, the maize *bm2* mutation, giving plants with lower lignin content and lower G content in lignins (Chabbert et al, 1994; Barrière et al, 2004c), was shown to occur in a MTHFR gene (Tang, 2011). Because the methionine pathway is located upstream to coniferyl and syringyl alcohol biosynthesis, a similar effect on G and S lignin unit of the MTHFR mutation was nevertheless expected. Considering the the S-adenosyl-L-methionine cycles, 16 genes were shown in the maize genome (Supplementary Table 5). Based on physical positions, there does not appear to be any tendency to clustering of methyltransferase genes and S-adenosyl-L-methionine cycle genes.

Genes involved in p-hydroxycinnamate acylation and transfer onto cell wall components

The acylation of S units in grass lignin occurs at the monolignol level and before the transfer of S-pCA in the cell wall space (Ralph et al, 1994; Lu and Ralph, 1999; Grabber and Lu, 2007; Martinez et al, 2008). This process of *p*-coumaroylation is therefore dependent upon the production of the activated *p*-coumaroyl-CoA (pCA-CoA) which is a key-compound in the phenylpropanoid pathway, and upon specific transferase(s) acylating sinapyl alcohol with an activated *p*-coumaric acid. The corresponding acyltransferases(s) was shown to use an activated acid (*p*-coumaroyl-CoA) to form the corresponding sinapyl *p*-coumarate (Hatfield et al, 2009), and a candidate enzyme with higher affinity towards sinapyl alcohol than towards coniferyl alcohol was also described (Martinez et al, 2008; Hatfield et al, 2009). The rice gene *Os01g18744* (OsPMT, Oryza sativa *p*-coumarate monolignol transferase), which belongs to the BAHD acyltransferase family, was recently shown to encode an enzyme catalyzing the acylation of monolignols via the activated *p*-coumaroyl-CoA (Withers et al, 2012). Moreover, this gene was specific to grass species, and co-expressed in rice with genes of the monolignol pathway (Mitchell et al, 2007). A set of OsPMT orthologs were shown in maize, with two close orthologs and several other orthologs that were also previously found as orthologs of genes putatively involved in arabinoxylan feruloylation.

Results obtained with the maize *bm3* mutant strongly suggest that FA is not biosynthesized by a COMT-catalyzed methylation of a caffeic precursor. The disruption of the COMT gene 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). As was shown for S unit acylation by the *p*-coumarate, the formation of feruloylated compounds does not occur at the free acid level, but involve conjugates of the ferulic acid which are likely CoA-esters (Fry et al, 2000). Corroborating this fact, 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), allowing the formation of feruloyl arabinoxylan-trisaccharide (FAXX). A reaction between feruloyl-CoA and UDP-arabinose was also considered, giving a FA-Ara-UDP intermediate which could be transferred to the arabinoxylan chain in a reaction catalyzed by a feruloyl-arabinose-

UDP transferase (Buanafina, 2009). Finally, another even less probable hypothesis for ferulate biosynthesis in grasses has been considered from investigations in the *Arabidopsis* REF1 mutant plants, which have a reduced content in soluble sinapate esters. This mutant is affected in a sinapaldehyde dehydrogenase gene and the REF1 protein exhibited *in vitro* both sinapaldehyde and coniferaldehyde dehydrogenase activities (Nair et al, 2004). The formation of free sinapic and probably ferulic acids in *Arabidopsis* is thus catalyzed via the oxidation of the corresponding cinnamaldehydes. 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). However, their physiological role has not yet been determined. It is indeed not yet known if such an ALDH pathway is functional in maize and grasses for the cell wall linked ferulate metabolism.

Genes involved in arabinoxylan feruloylation were thus tentatively identified as acyltransferase (AcT) encoding 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 included members of the Pfam family PF02458 encoding CoA-dependent AcT including hydroxycinnamyl transferases. A study of gene deregulation in rice (Piston et al, 2010) supported the involvement of these putative feruloyl-transferases. Rice plants with an individually reduced expression of four members of this family had a reduced content of ester-linked ferulate in leaves and/or stems. As previously cited, maize orthologs of the rice PF02458 genes putatively involved in arabinoxylan feruloylation were for some of them the same as those found as orthologs of the rice gene involved in *p*-coumaroylation of S units. Acyltransferases enzymes share several conserved domains, a fact that could partly explain the close paralogs found in the maize genomes (D'Auria, 2006). Only 14 genes were shown in the maize genome (Supplementary Table 6) to be involved in *p*-hydroxycinnamate acylation and transfer onto cell wall components. This list is probably not exhaustive, and new genes or families are yet to be discovered.

Based on investigations in a transposon-tagging progeny of 12,000 plants, the *sfe* mutant (M04-21) was shown to have a low ferulate-ether phenotype in both leaves, sheathes, and stems, together with a lower lignin content (Jung and Philips, 2010). Moreover, dairy cows fed M04-21 sfe silage, which had similar lignin content but a 30% lower etherFA content than the control line, had a greater intake (+1.5 kg day^{-1}) and a higher milk yield (+2.3 kg day^{-1} FCM 3.5%) than cows fed the control W23 silage (Jung et al, 2011). The gene underlying the sfe mutation is not yet known, but its discovery would be one of the best ways to track specific mechanisms involved in lignin and feruloylated arabinoxylan cross-linking. In addition, breeding for divergent ester-linked diferulate (diFA) concentration in maize stalk pith tissues induced, after two cycles of divergent selection, a 16% difference in diFA content between the two selected populations, with significant effect on rumen cell wall degradability (Barros-Rios et al, 2012). This significant diFA variation after breeding suggested that diFA deposition in maize pith parenchyma cell walls is a highly heritable trait. However, the corresponding involved genes are still unknown, all the more so given that divergent selection affected esterified and etherified FA contents differently, supporting the hypothesis that the metabolisms of these cell wall components are separately regulated (Barros-Rios et al, 2012).

Finally, reducing arabinosyl transferase activities would seem to be a relevant strategy for reducing ferulate cross-linkages in the walls. The ferulic acid is esterified to the α-1,3 linked arabinofuranose. Rice *xax1* mutant plants are deficient in feruloylated arabinosyl residues on xylan chains. The *xax1* mutant plants exhibit an increased extractability of xylan and increased saccharification, probably as a consequence of a lower degree of diferulic cross-linkages (Chiniquy et al, 2012). Other genes compromise the grafting of arabinose on xylan chains, and consequently the ferulate cross-linkages, including UDP-arabinopyranose mutase (Konishi et al, 2011), may be considered as targets for increased saccharification in biofuel generation processes.

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 transfer of data and knowledge related to tissue patterning and lignification from dicotyledons or gymnosperms to grasses is difficult due to 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 (Terashima and Fukushima, 1993; Tomlinson, 1995). However, the emergence and evolution of lignified tracheids and vascular tissues was based for all vascular plants on the expression of a preexisting poly-phenolic pathway (Boyce et al, 2003), with lignin targeted deposition in different cell types. In addition, many results obtained in lignification genetics and genomics illustrated a large commonality in genes involved in cell wall carbohydrate and phenolic biosynthesis in all plant species. Orthologs of factors regulating lignin-related gene expression in woody species are therefore likely candidate genes for reg-

ulation of maize lignin biosynthesis and deposition, even if the targets have possibly changed during plant evolution. Moreover, with the possible selection of grass specific genes, as has been shown for genes involved in arabinoxylan biosynthesis, specific grass transcription factors might have emerged during plant speciation and evolution.

MYB transcription factors

The regulation of phenylpropanoid gene 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 many phenylpropanoid genes (Sablowski et al, 1994; Peter and Neale, 2004), even if other interaction mechanisms also exist (Uzal et al, 2008). Other pathways are also regulated by R2R3-MYB, and based on aspen data, only 12% of the R2R3-MYB encoding genes showed the highest level of transcript abundance in differentiating xylem (Wilkins et al, 2009).

Only ZmMYB31, ZmMYB42, and ZmMYB46 have been proven to be related to the secondary wall formation in maize. ZmMYB31 and ZmMYB42 both have a repressive effect on the expression of several genes of the lignin pathway (Fornalé et al, 2006; Sonbol et al, 2009; Fornalé et al, 2010; Gray et al, 2012), while ZmMYB46 has an activator effect on secondary wall biosynthetic genes (Zhong et al, 2011). Similarly, PvMYB4, orthologous to ZmMYB42, was shown to have a repressive effect on lignin pathway genes, resulting in reduced lignin and pCA content in *Panicum virgatum* plants over-expressing this gene (Shen et al, 2010). Other MYB factors putatively involved in the regulation of maize lignification have been searched for as orthologs of lignin-related R2R3 MYB genes described in eucalyptus [EgMYB1, (Legay et al, 2007; Legay et al, 2010), EgMYB2 (Goicoechea et al, 2005)], poplar [PtMYB4, PttMYB21 or PtrMYB021 (Patzlaff et al, 2003; Karpinska et al, 2004; Wilkins et al, 2009)], pine [PtMYB1, PtMYB8 (Bomal et al, 2008)], barley [MYB hv5 and hv33, (Wissenbach et al, 1993)], and *Arabidopsis* [AtMYB46, AtMYB83, (Zhong et al, 2007; Zhong and Ye, 2012) AtMYB4, AtMYB7, AtMYB32 (Zhong and Ye, 2009; Zhou et al, 2009; Zhong and Ye, 2010; Zhong et al, 2010; Zhong and Ye, 2012), AtMYB20, AtMYB58, AtMYB63, AtMYB85 (Zhong et al, 2007; Zhou et al, 2009; Ohman et al, 2013), AtMYB52, AtMYB54, AtMYB69 (Zhong et al, 2008), AtMYB61 (Newman et al, 2004), AtMYB75 (Bhargava et al, 2010), and AtMYB103 (Ohman et al, 2013)]. In addition, a large overview of the R2R3-MYB gene family in maize has been recently proposed, with a comprehensive classification of all family members, including subgroups involved in regulation of lignified cell wall biosynthesis and deposition (Du et al, 2012). Maize MYB orthologous to AtMYB4, which was shown to be a negative regulator of lignin gene expression, were gathered in the G4 subgroup "phenylpropanoid pathway" (Du et al, 2012). This group included the two *ZmMYB31* (GRMZM2G050305) and *ZmMYB42* (GRMZM2G419239) genes, and members of this group are also orthologs of EgMYB1 and hv5 MYB. ZmMYB31 was shown with 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 (Fornalé et al, 2006; Sonbol et al, 2009). Orthologs of AtMYB58 and AtMYB63, which are known for their activating role in lignin biosynthesis (Zhou et al, 2009), were gathered in the G3 subgroup "lignin biosynthesis", and in the G2 subgroup with genes considered to be involved in "defense" processes. Orthologs of AtMYB85, which is involved in cell wall thickening and lignin deposition (Zhong et al, 2008), were classified in the G8 subgroup "lignin deposition". Similarly, orthologs of AtMYB52, AtMYB54 and AtMYB69, which also have a role in cell wall thickening and lignin biosynthesis in *Arabidopsis* (Zhong et al, 2008), were gathered in the G21 subgroup. EgMYB2, AtMYB46, AtMYB83, which were shown to be transcriptional activators of lignification, and the only ZmMYB146 maize ortholog were classified by Du et al (2012) in the G31 "metabolism" subgroup, together with PtMYB4 [ZmMYB146 (or GRMZM2G052606, bin 10.03) is also named ZmMYB46 by Zhong et al (2011)]. The latter subgroup of *AtMYB46/EgMYB2* genes was unexpectedly not related to any lignification process by Du et al (2012), despite their well known effect as an activator of seconday wall biosynthesis. In *Arabidopsis*, the two *AtMYB46* and *AtMYB83* genes, together with their NAC regulators and their direct targets, were indeed shown to be master genes of the secondary wall assembly. This set of genes regulates an array of downstream genes and thereby activates the secondary wall lignin and carbohydrate biosynthetic programs, in a multileveled feed-forward loop regulatory structure (Zhong and Ye, 2012). Other ZmMYB were considered as putative orthologs of *AtMYB61*, a gene that produced ectopic lignification when overexpressed in *Arabidopsis* plants (Newman et al, 2004). The latter were classified in the G13 "metabolism" subgroup (Du et al, 2012), which included the hv33 MYB gene expressed in lignifying tissue of barley (Wissenbach et al, 1993). In addition, the *ZmMYB130* gene was the only maize MYB belonging to the G28 subgroup "phenylpropanoid pathway", and the closest *Arabidopsis* ortholog of *ZmMYB130* is *AtMYB5*, a gene involved in anthocyanin metabolism. The *AtMYB75* gene, also assigned to the G6 "anthocyanin biosynthesis" subgroup by Du et al (2012), was shown to have a role in stem lignifica-

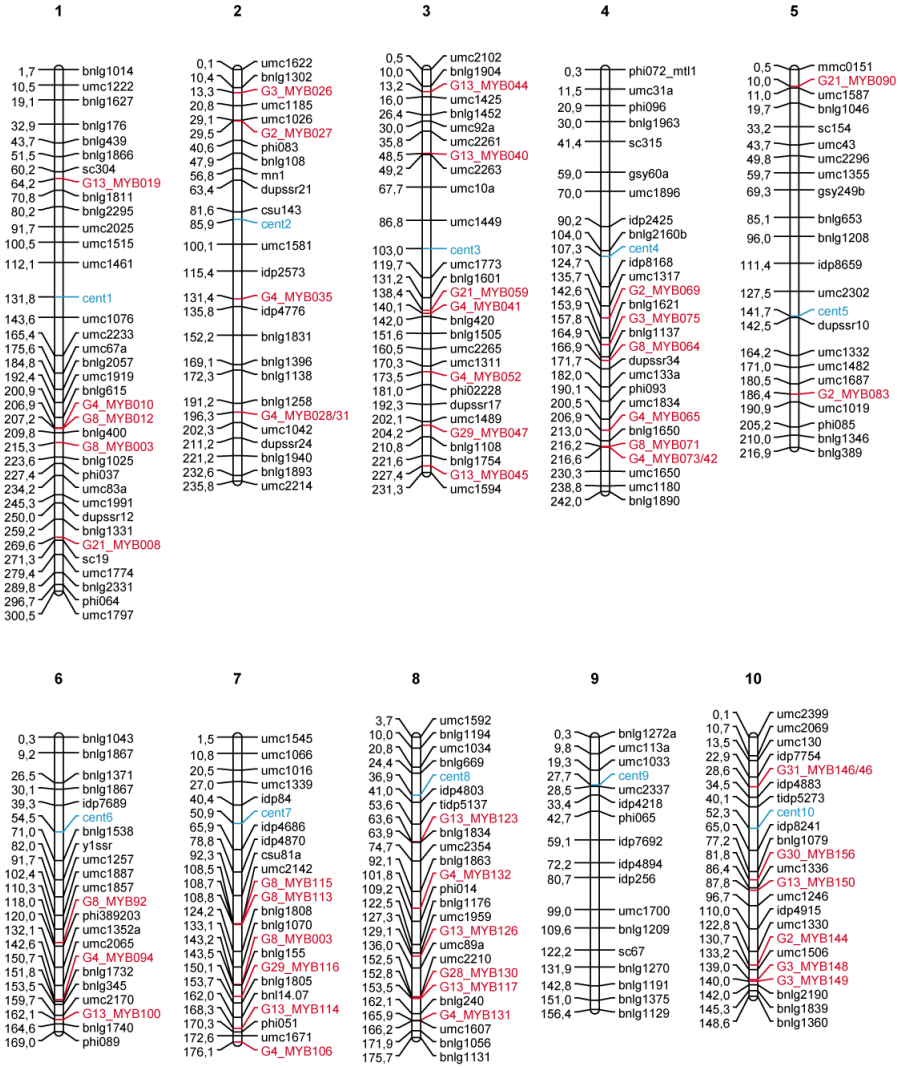

Figure 2 - Physical map of maize MYB genes putatively involved in maize secondary wall assembly [ZmMYB numbers and groups are given according to Du et al (2012)].

tion (Bhargava et al, 2010). It was thus considered to be "the first transcription factor reported so far that functions as a repressor of the entire secondary cell wall program" in *Arabidopsis* (Zhao and Dixon, 2011). In fact, while AtMYB75 was shown to be a positive regulator of anthocyanin accumulation, its physical interaction with KNAT7 was shown to increase the repressive effect of KNAT7 on secondary wall deposition (Bhargava et al, 2013). It is still unclear whether an ortholog of AtMYB75 exists or not in maize, but this group could be absent in grass genomes (Du et al, 2012). On the contrary, AtMYB103 was shown to positively and specifically regulate F5H expression in the lignin pathway, even if other roles in the cell wall assembly could not be ruled out (Ohman et al, 2013). The only maize ortholog of AtMYB103, located in bin 10.03, is also the only maize one belonging to the G30 group (Du et al, 2012), with genes involved in "cell wall thickening". Unexpectedly, most *Arabidopsis* MYB genes described as involved in the regulation of secondary wall assembly are activator factors, while most of those currently described in maize are repressor factors. Finally, 45 MYB transcription factors putatively involved in the secondary wall assembly were thus found in the maize genome (Figure 2, Supplementary Table 7). This list is possibly incomplete because most of them were highlighted as orthologs of non-grass plant MYB.

NAC transcription factors

Lignin and cell wall genes are regulated upstream the MYB transcription factor level. The first demonstrations that NAC transcription factors were involved in secondary wall assembly were likely the roles of NST1 and NST2 (NAC SECONDARY WALL THICK-

ENING PROMOTING FACTORS) in secondary wall thickening (Mitsuda et al, 2005) and the roles of VND6 and VND7 (VASCULAR-RELATED NAC-DOMAIN) in vessel xylem formation (Kubo et al, 2005). Over-expression of NST1, NST2 and SND1 thus induced ectopic lignified secondary cell wall thickenings in various *Arabidopsis* tissues (Mitsuda et al, 2005; Ko et al, 2007; Mitsuda et al, 2007; Zhong et al, 2006). Later, different *Arabidopsis* NAC proteins were shown to be master actors regulating the expression of several transcription factors (including MYB and other NAC genes) and/or genes involved in secondary cell wall biosynthesis (Zhong and Ye, 2007; Yamaguchi et al, 2008; Zhong et al, 2008; Zhong and Ye, 2009; Zhou et al, 2009; Zhong et al, 2010). NAC factors thus include NST 1-3 (with NST3 = SND1) which are specific to fibers, and the VND 1-7 which are specific to vascular vessels (Grant et al, 2010; Grima-Pettenati et al, 2012). Both NST and VND factors belong to the same NAC subfamily (Yamaguchi, 2010) and function as transcriptional activators. In addition, VNI2 (VND-INTERACTING2, and to a lesser extent VNI1) is a transcriptional repressor of vessel-specific genes regulated by VND7 (Yamaguchi et al, 2010). Interactions mostly occur between VNI2 and VND7, but also exist to a lesser extent with other VND proteins and possibly other NAC factors. In maize, four orthologs of *Arabidopsis* master NAC (ZmSWN2, 3, 6, and 7) were investigated and additional analysis has demonstrated that the latter ZmSWNs were functional orthologs of SND1 capable of activating the secondary wall biosynthetic program (Zhong et al, 2011). In addition, ZmSWN7 corresponds in fact to two closely duplicated genes in positions 28.04 and 28.14 Mbp on chromosome 9. Based on this set of data, 38 secondary wall NAC genes were shown in the maize, while only 11 were shown by Yao et al (2013), with the same limits as for MYB genes due to the search for non-grass plant orthologs (Supplementary Table 7).

Partner and target genes of MYB or NAC transcription factors

The class II *KNAT7 Arabidopsis* gene, first described as *IRX11* (Brown et al, 2005), is one of the direct targets of SND1, VND6 and AtMYB46 (Zhong et al, 2008; Ko et al, 2009). The *KNAT7* gene was later shown to be a transcriptional repressor of secondary cell wall biosynthesis, in interaction with OFP1 and OFP4 (OVATE FAMILY PROTEIN 1 and 4, Li et al, 2011b), and AtMYB75 (Li et al, 2012; Barghava et al, 2013). *OFP4* loss-of-function mutants also have an irregular xylem phenotype and thinner interfascicular fiber cell walls (Li et al, 2011b). *Arabidopsis* KNAT7 was considered to be involved in "a negative feedback loop within these regulatory networks governing secondary cell wall biosynthesis, working antagonistically to NAC and MYB positive regulators" (Li et al, 2012). In poplar, the class-I KNOX homeobox gene, ARBORKNOX2 (ARK2), which is ortholog to BREVIPEDICELLUS (or KNAT1) in *Arabidopsis*, is involved in terminal cell differentiation during secondary growth and subsequent lignin and cellulose contents (Du et al, 2009). Protein-protein interactions in the phenylpropanoid metabolism also involve at least basic helix-loop-helix (bHLH), and WD40 proteins (Grima-Pettenati et al, 2012). bHLH transcription factors belong to a protein family for which many different functions have first been identified in animals, including the control of cell proliferation and development of specific cell lineages. In the *Arabidopsis* and rice genomes, 133 and 167 bHLH genes have been shown (Heim et al, 2003; Li et al, 2006), respectively, likely indicating that more than 200 are present in the maize genome. Based on bioanalysis investigations, the rice bHLH proteins can potentially participate in a variety of combinatorial interactions, endowing them with the capacity to regulate a multitude of transcriptional programs related to plant cell and tissue development as well as plant metabolism (Li et al, 2006), with likely only a few members involved in lignified tissue assembly. WD40 proteins are regulatory proteins which contain a domain of nearly 40 amino acids often terminating with tryptophan (W) and aspartic acid (D) These specific traits give them their "WD40" denomination (Ramsay and Glover, 2005). Protein complexes composed of MYB and bHLH transcription factors associated with WD40 proteins have been shown to initiate multiple cellular differentiation pathways in a range of plants (Ramsay and Glover, 2005). Coherent models of the network of interactions that lead to diverse cell fates through the activity of this protein complex were considered as one basis of flexibility in plant morphology, and consequently to have likely played a major role in angiosperm evolution and success. The complex appears to have arisen in the land plant lineage, although its component parts are considerably more ancient (Ramsay and Glover, 2005). It was thus hypothesized that such complexes are also involved in controlling of the regulation of lignin-related R2R3-MYB transcription factors, all the more so given that the role of such complexes has been shown for regulation of anthocyan biosynthesis (Zhao et al, 2008; Brueggemann et al, 2010), including in maize (Cone et al, 1993; Grotewold et al, 2000). As a whole, 28 partners and targets of MYB genes were shown in the maize genome (Supplementary Table 7).

Zinc finger regulation factors putatively involved in lignified tissue assembly

Zinc finger proteins constitute one of the largest families of transcription factor regulatory proteins. They are involved in many regulations during plant development, including lignified tissue assembly. Zinc-finger C2H2 genes were the most frequently represented transcription factors in eucalyptus secondary xylem libraries (Rengel et al, 2009). The AtC3H14 zinc finger protein has been shown to activate all of the secondary wall phenolics and carbohydrate re-

lated genes tested. Both SND1 and AtMYB46 proteins were shown to bind to the AtC3H14 promoter, and AtC3H14 might function as master regulator of secondary wall biosynthesis, located downstream of AtMYB46 (Ko et al, 2009; Kim et al, 2012). Moreover, an AtC3H14 gene was shown to be a probable candidate gene underlying cell wall degradability QTLs in the Bur0 x Col0 progeny (Chavigneau et al, 2012). In addition, DOF type (DNA-binding with one finger) domain proteins, which are plant-specific zinc finger transcription factors involved as transcriptional activators or repressors in diverse plant growth and development processes (Yanagisawa, 2004; Kushwaha et al, 2011), are also involved in lignified tissue assembly. The high cambial activity of the HCA2 *Arabidopsis* mutant resulted from an elevated expression of a DOF transcription factor (AtDOF34) preferentially expressed in the cambium, phloem, and interfascicular parenchyma cells of stems (Guo et al, 2009). Ectopic lignification was also related to variation in DOF gene expression in *pom1*, *eli1* (*ectopic lignification 1*) and *det3* (*de-etiolated 3*) *Arabidopsis* mutants, in addition to expression variation of MYB genes (Rogers et al, 2005). As DOF type zinc finger, C3HC4 type RING zinc finger proteins also have important roles during plant growth and tissue assembly (Ma et al, 2009). Finally, LIM zinc finger proteins can also be involved in the regulation of plant lignification, as shown with the tobacco NtLIM1 acting as a positive regulator of the lignin pathway (Kawaoka and Ebinuma, 2001). LIM proteins are characterized by zinc-binding domains that ligate two zinc ions. Unlike the classical zinc fingers, these domains do not bind DNA, but mediate interactions with other proteins (Matthews et al, 2009). WRKY zinc finger proteins have highly conserved WRKYGQK amino acid sequences in their N-terminal part, followed by the C2H2 or C2HC zinc-finger motifs. WRKY proteins are involved in diverse physiological and developmental processes, especially including defense against biotic stresses (Wei et al, 2012). However, their role in cell wall constitutive lignification has not yet been established (Wu et al, 2005; Guillaumie et al, 2010; Rushton et al, 2010; Tripathi et al, 2012). Nonetheless, the AtWRKY12 (At2g44745) gene was highly expressed in lignifying stems (http://genecat.mpg.de/database), and its mutation induced secondary wall formation of pith cell (Wang et al, 2010). Similarly, the grapevine transcription factor WRKY2 was shown to be specifically expressed in cells undergoing lignification in young grapevine stems (Guillaumie et al, 2010). According to the Wei et al (2012) classification of maize WRKY genes, orthologs of VvWRKY2 and AtWRKY12 belonged to subgroups I and IIc, respectively. In addition, 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).

Other genes and regulation factors putatively involved in secondary wall assembly

The *Arabidopsis COV1* (continuous vascular ring) gene encodes an integral membrane protein of unknown function which is supposed to be involved in a mechanism that negatively regulates the differentiation of stem vascular tissue by a mechanism independent of auxin (Parker et al, 2003). In addition to the firstly described *COV1* gene (At2g20120), two COV1-like paralogs were later identified as the *LCV2* and *LCV3* genes [Like-COV-2 (At1g43130) and Like-COV-3 (At2g18460), TAIR database (http://arabidopsis.org/)]. Ten orthologous genes were found in the maize genome, including four, five, and one ortholog for *COV1*, *LCV2*, and *LCV3*, respectively. Colocalizations of cell wall degradability QTLs were shown with seven COV-like genes (out of nine), the only maize ortholog of *COV LCV3* being one of the non-colocalizing genes (unpublished data). In addition, one of the *LCV2* 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. The two latter facts likely corroborated the involvement of COV-like genes in maize and grass cell wall assembly.

Members of a small class III homeodomain-leucine zipper family, including AtHB8, AtHB9 (PHAVOLUTA), AtHB14 (PHABULOSA), AtHB15 (CORONA), and IFL1 (REVOLUTA), are expressed in vascular tissues and they have been considered to play regulatory roles in vascular differentiation (Talbert et al, 1995; Ratcliffe et al, 2000; Baima et al, 2001; McConnell et al, 2001; Green et al, 2005). The *IFL1 Arabidopsis* gene has two maize orthologs, the mutants of which have rolled leaf phenotypes (*RLD1* and *RLD2*). The maize *RLD1* gene is regulated by the Zm-miR166 miRNA (Juarez et al, 2004). The expression of the aspen *PtaHB1* gene, which is also orthologous to IFL1, is also inversely correlated with the level of miR166 miRNA (Ko et al, 2006). In addition, interactions between HDZIP III and KANADI gene family members were shown to be involved in the establishment of the spatial arrangement of phloem, cambium and xylem. It was considered that HDZIP III and KANADI transcription factors control cambium activity, with KANADI proteins acting on auxin transport, and HDZIP III proteins promoting axial cell elongation and xylem differentiation (Ilegems et al, 2010). Corroborating this assertion, the down-regulation of the poplar class III HD-ZIP gene *PtrHB7* led to plants displaying significant changes in vascular tissues with a reduction in xylem and an increase in phloem. On the contrary, *PtrHB7* over-expression enhanced differentiation of cambial cells toward xylem cells and inhibited phloem differentiation (Zhu et al, 2013). Transcrip-

tional analysis revealed that genes regulating xylem and phloem differentiations were correspondingly up- or down-regulated (Zhu et al, 2013). The *PtrHB7* gene has six close orthologs in maize, including the two *RLD1* and *RLD2* genes. In addition, interactions between NAC and zinc finger homeodomain proteins have been reported (Tran et al, 2006).

The GRAS SCARECROW and SCARECROW-like proteins belong to a plant-specific transcription factor family which contains basic leucine zipper regions and are involved in complex regulatory pathways regulating tissue patterning and differentiation (Di-Laurenzio et al, 1996; Lee et al, 2008). SCARECROW proteins are thus involved in bidirectional cell signaling mediated by miRNA165/166. These proteins interfere with the transcription factor SHORT ROOT (SHR) equally expressed in stem and root, and HDZIP III proteins, towards the control of xylem patterning (Carlsbecker et al, 2010). These genes could be considered as candidates inducing cell wall degradability variation even if they are involved upstream in the pathway.

ROP family of Rho-like GTPases are important signalling proteins during plant growth and tissue differentiation, with very little data related to their role in xylogenesis. A member of the plant ROP family (EgROP1) was shown to be preferentially expressed in the cambial zone and differentiating xylem of eucalyptus (Rengel et al, 2009). Its over-expression in *Arabidopsis* altered vessel formation and fibre growth in secondary xylem, with changes in secondary cell wall thickness, lignin and xylan composition (Foucart et al, 2009). ROP/RAC/RAB genes encode geranylgeranylated GTP-binding proteins (GTPases) involved in the auxin proteolysis pathway. The latter are thought to provide a universal mechanism in the control of extracellular signal transmission to intracellular metabolic pathways related to growth, differentiation, development and defense responses (Gu et al, 2004; Nibau et al, 2006). Several of them are involved in autophagy and xylem development (Kwon et al, 2010). Consequently, the latter could be considered as putative candidates.

Candidate genes were also considered in the ERF/AP2 (ethylene responsive factor/APETALA2) SHINE family. After over-expression investigations in rice, an ERF/AP2 gene was considered as an upstream transcriptional regulator of both master and secondary target genes involved in the biosynthesis of cell wall phenolic and carbohydrate components. This ERF/AP2 transcription factor was supposed to directly bind promoter regions of NAC and MYB genes involved in regulation of cell wall assembly. Rice plants over-expressing *Arabidopsis* SHINE2 gene thus had lower lignin and higher cellulose and hemicellulose contents, without changes in plant strength or overall performances (Ambavaram et al, 2011).

The *Arabidopsis* SHP1 (SHATTERPROOF MADS-box) gene, which has been shown to specify with SHP2 the lignified valve margin of mature siliques (Liljegren et al, 2000), likely has other roles in tissue lignification as it is also expressed in stems and down-regulated at the maturing stage (Ko and Han, 2004). While the roles of their maize orthologs are not known, the maize *ZmZAG5* gene was under-expressed to nearly the same level as the disrupted COMT gene in *bm2* plantlets, while it was 3.5 times over-expressed in the ear internode of *bm3* silking plants (Guillaumie et al, 2007b; Guillaumie et al, 2008).

The maize α-expansin 5 (*EXPA5*) gene also probably has a function of interest in cell wall metabolism. Most expansins are involved in the disruption of hydrogen bonds between cellulose microfibrils and cross-linking hemicelluloses in the wall, restoring the long-term extension to cell walls (Li et al, 2003c). However, roles of expansins that do not involve wall expansion have already been shown. Expansins have been associated with the growth of protoxylem elements in Zinnia stems (Im et al, 2000). Similarly, several expansins appeared to be expressed during the differentiation of the tracheary elements, and the *ZmEXPA5* gene was expressed in leaf region where secondary cell wall deposition occurred (Milioni et al, 2001; Muller et al, 2007). The latter facts suggest their possible involvement in secondary wall formation. While ten genes are annotated α-expansin in the maize sequence database and nine of them have numerous paralogs, the *EXPA5* gene (GRMZM2G361064) appears different from the others as it is the only one without any paralogs. Moreover, the *EXPA5* gene is located under a major cell wall QTL in bin 6.06 (Courtial et al, 2013) and it was at least 20 times more expressed in four RILs with high cell wall degradability than in the parental line with low cell wall degradability (Courtial et al, 2012).

The fasciclin-like arabinogalactan (FLA) proteins are characterized by a juxtaposition of glycosylated arabinogalactan-protein (AGP) domains and one or two fasciclin (FAS) putative cell-adhesion domains. Members of this gene family are implicated in many developmental roles, even if their functions remain largely undefined. However, among the 21 FLA *Arabidopsis* genes, the two *AtFLA11* (or *IRX13*) and *AtFLA12* genes were shown to be involved in secondary wall formation (Persson et al, 2005; MacMillan et al, 2010), with high transcript abundance in stem cells undergoing secondary-wall deposition. In addition, (double) mutant plants had altered stem biomechanics, altered cell wall architecture and composition, lower cellulose contents, and higher lignin contents. Other results obtained in their orthologs in poplar (Lafarguette et al, 2004), Zinnia (Dahiya et al, 2006), and eucalyptus (Qiu et al, 2008) strengthened the involvement of *AtFLA11/12*-like genes in secondary wall formation. In addition, another FLA gene, *AtFLA4*, was shown to be required for normal cell expansion, and the mutant *Salt Overly Sensitive5* (*SOS5*) have thinner

cell walls (Shi et al, 2003).

Finally, *Arabidopsis* MAP70 microtubule-associated proteins were shown to be essential for defining where secondary cell wall polymers were positioned and for determining the overall pattern of xylem vessel secondary cell walls (Pesquet et al, 2010; Pesquet et al, 2011).

This set of miscellaneous genes and regulation factors putatively involved in secondary wall assembly included 87 genes (Supplementary Table 8), which likely are of varying importance in cell wall assembly, and correlatively in cell wall degradability variation. SHATTERPROOF, HDZIP and COV genes can be considered of great importance in lignified tissue patterning, and thus could be relevant breeding targets to drive lignification in areas where this trait is essential for plant standability and disease or pest tolerance.

Questioning the role of miRNA in cell wall and lignified tissue biosynthesis

The role of micro-RNA in regulation of plant lignified tissue assembly is little documented. Only a few miRNA have been shown to be involved or are thought to be involved in secondary cell wall formation. The miR166 and 165 families are well known for this developmental processus. The two miR165 and miR166 have been shown to target and regulate the transcription HD-ZIP III genes, including those involved in vascular tissue differentiation (Juarez et al, 2004; Kim et al, 2005; Ko et al, 2006). The maize ZmmiR166 miRNA thus accumulates in phloem and regulates the maize *rolled-leaf1* gene (*RLD1*), which encodes an HD-ZIP III transcription factor. The *Arabidopsis* ortholog of *RLD1* is the IFL1/REVOLUTA gene, which is similarily involved in the differentiation of interfascicular fibers and secondary xylem (Ratcliffe et al, 2000; Juarez et al, 2004). Moreover, 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; Zhang et al, 2009). In addition, miR164, as well as miR397, miR408, and miR528, were shown to target laccase genes (Zhang et al, 2009). Some of the latter are potentially involved in monolignol polymerization in the cell wall through the regulation of copper homeostasis (Abdel-Ghany et al, 2008). miR171, which targets SCARECROW genes (Zhang et al, 2009), could also be a possible candidate involved in variation of cell wall degradability. Finally, small interfering RNA derived from the 3'-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).

The final candidate gene list

Based on the different considered functions required for the secondary cell wall assembly, 409 putative candidate genes were shown in the maize genome (Supplementary Tables 1-8), out of which 130 were involved in phenolic compound biosynthesis, 81 were involved in cell wall carbohydrate biosynthesis, and 198 were involved in regulation mechanisms. This candidate gene list, which focused on genes known to be involved in cell wall component biosynthesis and regulation, cannot be considered as complete. Other genes, whose roles in cell wall lignification and deposition have not yet been defined, should very likely be added to the list of candidates with a required actictity in secondary cell wall assembly. Genes encoding proteins of still unknown function should be added to the list, as several of them are probably also involved in lignified tissue biosynthesis and deposition. As observed for all genes (Schnable et al, 2009), only a few genes related to cell wall biosynthesis and assembly were located around centromeres, and most of the latter were located in proximal and distal positions of chromosomes. When considering successive 30 Mbp long intervals all along chromosomes, a higher number of cell wall related genes were observed in the 0 - 30 Mbp areas of chromosomes 2 and 5. Similarly, a large number of cell wall genes were observed in the distal areas of chromosomes 5, 6, and 8 (Figure 3). Gene duplications in tandem positions were observed for several family members including especially PAL in bins 2.03, 4.06, and 5.05 (2, 3, and 4 genes, respectively), ZRP4-like OMT in bins 2.05, 6.06, and 9.04 (2, 4, and 2 genes respectively), or glycosyl transferase IRX10-like in 3.05, 6.07, and 8.06 (3, 2, and 2 genes, respectively).

Discussion and conclusion

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. A high biomass yield alone will certainly lead to disappointing results in dairy cow feeding, with reduced milk yields and/or the necessicity of an extra cattle feeding with expensive concentrates due to the lower silage intake, digestibility and energy value. Similarly, for biofuel production, low degradable biomass will incur greater transport, processing and fermentation costs. However, fermentation costs could be expected to be lower if acid, alkaline, and/or heat pretreatments were used in biogas or bioethanol processes. But such treatments are both expensive and not environmentally friendly. Thus from an environmental and economic point of view, it is essential to breed more degradable plants, as well as plants with cell walls more susceptible to pretreatment processes. Using strategies allowing the introduction of alternative monomers (aldehydes, ester conjugates, …) at reasonably low levels will not greatly alter the structural properties, as has been observed in maize bm1 or sorghum *bmr6* plants. But these modifications will render the lignin polymer much easier to cleave into smaller fragments during pretreatments

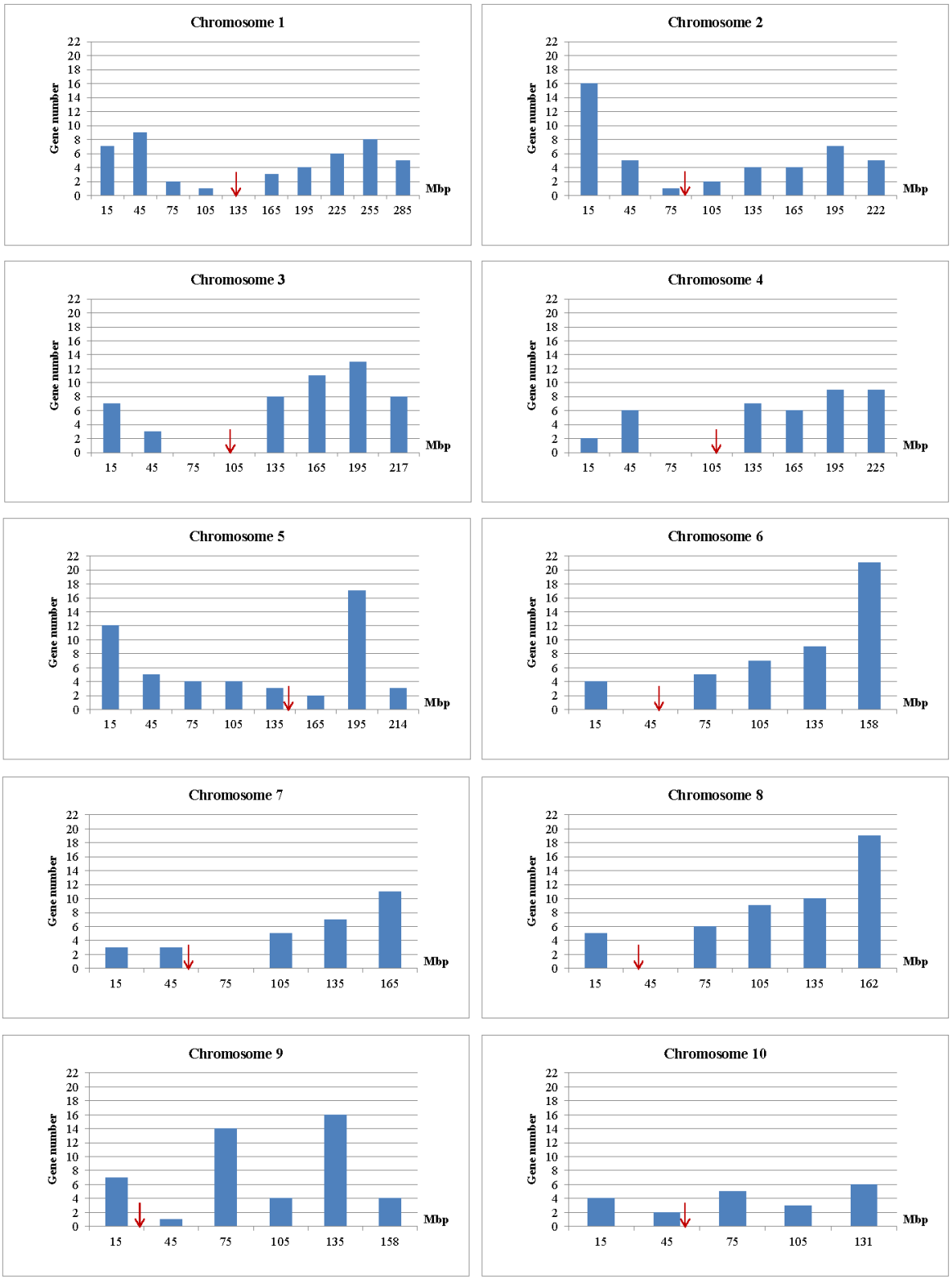

Figure 3 - Cell wall related genes distribution along maize chromosomes in 30 Mbp interval length (centromer positions are indicated by vertical arrows).

(Vanholme et al, 2012). Therefore, there is little doubt that cell wall qualities should be among the major targets for the improvement of silage and biofuel grass maize varieties. However, breeding grain maize varieties for the specific ability of their straw to be used as a bioproduct and 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 maize plants.

Thanks to the huge advances in genetic and genomic tools and knowledge, maize is also an inescapable model plant for grass secondary wall lignification and degradability studies, and consequently for forage and biofuel production improvements. 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 short-cycle model C3 grass *Brachypodium distachyon* (Garvin, 2007), and the recently sequenced and proposed as model plant for C4 grass *Setaria italica* (Bennetzen et al, 2012; Zhang et al, 2012) will both be complementary resources for gene mining and validation. The most important current limitation when it comes to using maize as a model system in plant genomics is the frequency of repeated elements, which represented between 75 and 80% of the genome, and the presence of several hundreds of gene copy number variations (CNV) together with several thousands of presence-absence sequence variations (PAV) in the maize genome (San Miguel et al, 1998; Meyers et al, 2001; Schnable et al, 2009; Springer et al, 2009; Belo et al, 2010; Lai et al, 2010; Swanson-Wagner et al, 2010). As a consequence, despite the fact that high throughput sequencing technologies are available, sequence comparison between lines is made difficult due to the difficulty of contiguing the obtained short-read sequence.

Among plant polymers, lignins have metabolic plasticity, with variable structures and non-conventional monomer incorporation, giving large variation in cell wall properties for which the underlying genetic determinants 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, high standability, 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, that should be more hardy and drought tolerant than those of the previous decade, due to climatic changes.

Despite the fact that genes involved in cell wall carbohydrate and phenolic component biosynthesis have been listed, together with genes involved in their regulation, only a (very) few can be currently considered to be the relevant determinants of main variations in cell wall degradability. Discovery of the relevant candidate genes involved in genetic variation of cell wall degradability, and to be used in marker assisted selection, will be based on a set of further investigations. One of the most promising investigative methods will probably be based on studies of co-localizations between cell wall trait QTLs and cell wall related genes, taking into consideration genes and QTL physical positions. Investigations in the F288 x F271 RIL progeny have highlighted the possible role of *ZmMYB42* and a *COV1*-like gene for two cell wall QTLs located in bin 4.09 (Courtial et al, 2012). Similarly, three MYB, the 4CL2, and the CCR1 genes are possible candidates for the cluster of cell wall QTLs shown in bin 1.07, in the F838 x F286 RIL progeny (Barrière et al, 2008). In the same progeny, the *ZmMYB46* is in close position to the cluster of QTLs located between bin 10.02 and 10.04. In contrast, no obvious candidate genes have yet been shown to explain numerous previously shown QTLs. The latter fact supports the possible role of genes of unknown function, of genes with known function but not yet related to cell wall assembly, as well as the probable role of non-coding sequence as the relevant determinants of variations in cell wall quality traits. Reverse genetics and transposon tagging, together with QTL fine mapping, are complementary essential strategies to understand the major traits involved in plant cell wall degradability variations.

In addition, the frequent clustering of QTLs for cell wall related traits raised the question of whether the underlying genetic determinant corresponds to a unique factor, or to a small set of highly linked and co-regulated genes. Colocalizations between cell wall degradability, core lignin content, and syringaldehyde QTLs could correspond to a shared mechanism involved in lignin biosynthesis and duration of lignin deposition. In addition, a greater proportion of S units in lignins could also correspond to a polymer richer in β-O-4 linkages and thus more linear, with greater masking effects on carbohydrates. Colocalizations between cell wall degradability, etherFA, and diFA QTLs could correspond to other genetic mechanisms involved in FA biosynthesis, in cross-linkages between arabinoxylan chains and between arabinoxylans and lignins. Occurrences of the simultaneous colocalizations between cell wall degradability, lignin content and structure, and ferulate related trait QTLs complicate the understanding and identification of the possible underlying genetic determinant(s). A cluster of linked genes involved in the different mechanisms of cell wall biosynthesis and assembly is likely the simplest situation to consider, but a single co-regulating "master" factor located upstream in the pathway of cell wall assembly can also be considered. Depending on colocalizing traits and QTLs, the two types of situation probably coexist in the maize genome. The fact that different genomic determinants are involved in cell wall degradability variation and are linked in close or identical positions strengthens the possibili-

ties of breeding for higher values of this trait without negative effects on agronomic value (yield, biotic and abiotic stress tolerance). Nevertheless, marker-assisted selection is essential in order to correctly identify the favorable recombinations, which require prior identification of the genes involved. Gene identification is all the more important given that there is often great gap in agronomic value between lines of interest for feeding value traits and elite modern lines. Finally, 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 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 on cell wall traits in unused accessions, old lines, and exotic resources, in order to discover new mechanisms or alleles allowing significant wall degradability improvement, without (too) negative effects on yield and agronomic value.

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Supplementary table 1 - List of putative maize candidate genes involved in cell wall biosynthesis and assembly. Cellulose related genes.

Gene names	Gene number	Chr	Pos Mbp	Bin
Brittle stalk 2 COBRA-like	GRMZM2G109326	9	123,24	9.04
Cellulose synthase 11 (CesA11a)	GRMZM2G037413	2	29,35	2.03
Cellulose synthase 11 (CesA11b)	GRMZM2G055795	3	198,37	3.07
Cellulose synthase 12 (CesA12)	GRMZM2G142898	7	117,51	7.02
Cellulose synthase-1 (CesA-1)	GRMZM2G112336	8	80,22	8.03
Cellulose synthase-2 (CesA-2)	GRMZM2G027723	6	128,56	6.05
Cellulose synthase-3 (CesA-3)	GRMZM2G039454	3	11,64	3.03
Cellulose synthase-4 (CesA-4)	GRMZM2G424832	7	18,61	7.02
Cellulose synthase-5 (CesA-5)	GRMZM2G111642	1	290,59	1.11
Cellulose synthase-6 (CesA-6)	GRMZM2G113137	1	296,25	1.12
Cellulose synthase-7 (CesA-7)	GRMZM2G025231	7	37,02	7.02
Cellulose synthase-8 (CesA-8)	GRMZM2G177631	7	26,49	7.02
Cellulose synthase-9 (CesA-9)	GRMZM2G018241	2	161,12	2.06
Chitinase-like POM1-CLT1-ELT1-CTL2-like	GRMZM2G168364	7	134,14	7.03
FRA1-like1 "kinesin motor region"	GRMZM2G026560	5	206,46	5.07
FRA1-like1 "kinesin motor region"	GRMZM2G334142	5	206,31	5.07
FRA1-like1 "kinesin motor region"	GRMZM2G026218	7	47,93	7.02
FRA2-like1 "AAA ATPase" ERH3	GRMZM2G054715	3	208,04	3.08
FRA2-like2 "AAA ATPase" ERH3	GRMZM2G017305	8	151,26	8.05
UDP sugar transporter (rice brittle stalk 14 Os02g40030-like)	GRMZM2G133226	4	138,15	4.06
UDP sugar transporter (rice brittle stalk 14 Os02g40030-like)	GRMZM2G02097	5	184,22	5.05
β-1,4-endoglucanase KORRIGAN-like	GRMZM2G147849	1	273,69	1.10
β-1,4-endoglucanase KORRIGAN-like	GRMZM2G110735	5	8,26	5.02

Supplementary table 2 - List of putative maize candidate genes involved in cell wall biosynthesis and assembly. Arabinoxylans related genes.

Gene names	Gene number	Chr	Pos Mbp	Bin
Feruloyl-AX β-1,2-xylosyl transferase (XAX1-Os02g22380-like, GT61)	GRMZM2G094579	4	239,48	4.10
Feruloyl-AX β-1,2-xylosyl transferase (XAX1-Os02g22380-like, GT61)	GRMZM2G098793	5	58,75	5.03
Feruloyl-AX β-1,2-xylosyl transferase (XAX1-Os02g22380-like, GT61)	GRMZM2G062552	5	76,43	5.03
Feruloyl-AX β-1,2-xylosyl transferase (XAX1-Os02g22380-like, GT61)	GRMZM2G354610	9	51,34	9.03
Feruloyl-AX β-1,2-xylosyl transferase (XAX1-Os02g22380-like, GT61)	GRMZM2G074896	5	68,42	5.03
Transferase xylan-O-3-arabinosyl transferase (XAT1-like, GT61)	GRMZM2G176576	3	166,69	3.06
Transferase xylan-O-3-arabinosyl transferase (XAT1-like, GT61)	GRMZM2G447347	6	70,29	6.01
Transferase xylan-O-3-arabinosyl transferase (XAT2-like, GT61)	GRMZM2G096946	4	219,6	4.09
Glycosyltransferase IRX8-like (GT8D)	GRMZM2G107854	1	25,42	1.02
Glycosyltransferase IRX8-like (GT8D)	GRMZM2G098434	3	124,64	3.04
Glycosyltransferase IRX8-like (GT8D)	GRMZM2G113506	9	147,36	9.06
Xylan-related (OsIRX10-like (orth1), IRX10-like, GT47)	GRMZM2G000581	8	160,34	8.06
Xylan-related (OsIRX10-like, IRX10-like, GT47)	GRMZM2G134308	8	160,39	8.06
Xylan-related (OsIRX10-like (orth1), IRX10-like, GT47)	GRMZM2G059825	6	162,55	6.07
Xylan-related (OsIRX10-like, IRX10-like, GT47)	GRMZM2G059845	6	162,56	6.07
Xylan-related (OsIRX10-like (orth1), IRX10-like, GT47)	GRMZM5G898668	3	157,02	3.05
Xylan-related (OsIRX10-like, IRX10-like, GT47)	GRMZM2G056702	3	157,05	3.05
Xylan-related (OsIRX10-like, IRX10-like, GT47)	GRMZM2G448834	3	157,08	3.05
Xylan-related (OsIRX10-like, IRX10-like, GT47)	GRMZM2G152029	5	6,04	5.01
Xylan-related (OsIRX10-like, IRX10-like, GT47)	GRMZM2G023020	2	62,92	2.04
UDP-arabinopyranose mutase (UAM1, Os03g40270-like)	GRMZM2G073725	1	248,86	1.08
UDP-arabinopyranose mutase (UAM1, Os03g40270-like)	GRMZM2G087326	5	18,77	5.03
UDP-arabinopyranose mutase (UAM2, Os04g56520-like)	GRMZM2G173341	2	3,81	2.01
UDP-arabinopyranose mutase (UAM3, Os07g41360-like)	GRMZM2G045287	7	164,24	7.05
UDP-D-glucose dehydrogenase (G6DH)	GRMZM2G328500	1	278,13	1.10
UDP-D-glucose dehydrogenase (G6DH)	GRMZM2G058244	1	249,42	1.08
UDP-D-glucose dehydrogenase (G6DH)	GRMZM2G862540	5	6,69	5.01
UDP-D-Glucuronic acid decarboxylase	GRMZM2G044027	1	41,47	1.03
UDP-D-Glucuronic acid decarboxylase	GRMZM2G381473	3	57,10	3.04
UDP-D-Glucuronic acid decarboxylase	GRMZM2G359234	3	181,70	3.06
UDP-D-Glucuronic acid decarboxylase	GRMZM2G007405	6	138,25	6.05
UDP-D-Glucuronic acid decarboxylase	GRMZM2G007195	8	87,04	8.03
UDP-D-Glucuronic acid decarboxylase	GRMZM2G165357	9	139,41	9.05
UDP-D-Glucuronic acid decarboxylase	GRMZM2G347717	9	138,88	9.05
UDP-D-xylose-4-epimerase (MUR4-like)	GRMZM2G000632	1	47,47	1.03
UDP-D-xylose-4-epimerase (MUR4-like)	GRMZM2G040397	2	9,50	2.02
UDP-D-xylose-4-epimerase (MUR4-like)	GRMZM5G830983	9	136,62	9.05
UDP-D-xylose-4-epimerase (MUR4-like)	GRMZM2G145460	10	142,59	10.07
Xylan glucuronosyltransferase (GUX1/GUX3, GT8)	GRMZM2G135743	3	172,05	3.06
Xylan glucuronosyltransferase (GUX1/GUX3, GT8)	GRMZM2G058472	6	147,97	6.05
Xylan glucuronosyltransferase (GUX1/GUX3, GT8)	GRMZM2G002023	8	165,23	8.06
Xylan glucuronosyltransferase (GUX2, GT8)	GRMZM2G109431	1	17,69	1.02
Xylan O-acetylation (Altered-Xyloglucan-4 AXY4 TBL27-like)	GRMZM2G340933	2	177,72	2.06
Xylan O-acetylation (Altered-Xyloglucan-4 AXY4 TBL27-like)	GRMZM2G039525	2	177,75	2.06
Xylan O-acetylation (Altered-Xyloglucan-4 AXY4 TBL27-like)	GRMZM2G131152	8	112,68	8.04
Xylan O-acetylation (Altered-Xyloglucan-4like AXY4like TBL22-like)	GRMZM2G004183	3	213,60	3.09
Xylan O-acetylation (Altered-Xyloglucan-4like AXY4like TBL22-like)	GRMZM2G107373	6	113,93	6.04
Xylan O-acetylation (Caps1 RWA1-4 like)	GRMZM2G463445	1	238,54	1.07
Xylan O-acetylation (Caps1 RWA1-4 like)	GRMZM2G076394	2	113,32	2.05
Xylan O-acetylation (Caps1 RWA1-4 like)	GRMZM2G370741	5	172,59	5.05
Xylan O-acetylation (Caps1 RWA1-4 like)	GRMZM2G458538	6	166,88	6.07
Xylan O-acetylation (Caps1 RWA1-4 like)	GRMZM2G020721	9	134,30	9.05
β-1,4-xylan synthase or xylosyltransferase (IRX14-like, GT43)	GRMZM2G113655	5	50,09	5.03
β-1,4-xylan synthase or xylosyltransferase (IRX14-like, GT43)	GRMZM2G150302	6	88,92	6.02
β-1,4-xylan synthase or xylosyltransferase (IRX9-like, GT43)	GRMZM2G001079	1	44,18	1.03
β-1,4-xylan synthase or xylosyltransferase (IRX9-like, GT43)	GRMZM2G012874	8	131,69	8.05
β-1,4-xylan synthase or xylosyltransferase (IRX9-like, GT43)	GRMZM2G118959	9	138,24	9.05

Supplementary table 3 - List of putative maize candidate genes involved in cell wall biosynthesis and assembly. Genes of the shikimate pathway.

Gene names	Gene number	Chr	Pos Mbp	Bin
3-Dehydroquinate synthase (Solanum, DHQ)	GRMZM2G573867	2	196,36	2.07
3-Dehydroquinate synthase (Solanum, DHQ)	GRMZM2G051129	7	175,83	7.05
3-Deoxy-D-arabinoheptulosonate-7-phosphate synthase (DAHP, DHS1/2-like)	GRMZM2G117707	7	166,47	7.05
3-Deoxy-D-arabinoheptulosonate-7-phosphate synthase (DAHP, DHS1/2-like)	GRMZM2G365160	2	212,18	2.08
5-Enolpyruvylshikimate-3-phosphate synthase (At2g45300, EPSP)	GRMZM5G877500	9	22,68	9.03
Adenosine kinase (At3g09820, ADK1)	GRMZM2G540538	5	186,52	5.05
Adenosine kinase (At3g09820, ADK2)	GRMZM2G135132	4	143,08	4.06
Adenosine kinase (At5g03300, ADK3)	GRMZM2G089767	2	28,14	2.03
Arogenate/prephenate dehydratase (ADT/PDT, AtADT1)	GRMZM2G466543	2	165,87	2.06
Arogenate/prephenate dehydratase (ADT/PDT, AtADT12)	GRMZM2G141273	1	43,75	1.03
Arogenate/prephenate dehydratase (ADT/PDT, AtADT12)	GRMZM2G125923	10	113,52	10.04
Arogenate/prephenate dehydratase (ADT/PDT, AtADT2)	GRMZM2G145451	9	138,33	9.05
Arogenate/prephenate dehydrogenase (ADH/PDH, At1g15710)	GRMZM2G084942	5	59,29	5.03
Arogenate/prephenate dehydrogenase (ADH/PDH, At1g15710)	GRMZM2G085117	5	59,30	5.03
Arogenate/prephenate dehydrogenase (ADH/PDH, At1g15710)	GRMZM2G365961	6	85,80	6.02
Arogenate/prephenate dehydrogenase (ADH/PDH, At1g15710)	GRMZM2G324297	9	61,36	9.03
Chorismate mutase (At1g69370, CM3)	GRMZM2G028369	3	194,55	3.06
Chorismate mutase (At3g29200, CM1)	GRMZM2G116087	8	173,11	8.08
Chorismate mutase (At5g10870, CM2)	GRMZM2G179454	5	92,19	5.04
Chorismate mutase (AtCM123, CM4)	GRMZM2G124365	8	173,10	8.08
Chorismate synthase (At1g48850, EMB1144)	GRMZM2G164562	1	35,46	1.03
Chorismate synthase (At1g48850, EMB1144)	GRMZM2G036861	9	141,94	9.05
Prephenate aminotransferase (Solanum, At2g22250, PAT)	GRMZM2G400604	3	174,37	3.06
Prephenate aminotransferase (Solanum, At2g22250, PAT)	GRMZM2G033799	8	101,41	8.03
Shikimate kinase (AtSK1, AtSK2)	GRMZM2G004590	2	6,49	2.01
Shikimate kinase (AtSK1, AtSK2)	GRMZM2G161566	4	181,73	4.07
Shikimate kinase (AtSK1, AtSK2)	GRMZM2G070218	5	207,71	5.07

Supplementary table 4 - List of putative maize candidate genes involved in cell wall biosynthesis and assembly. Monolignol biosynthesis, transport, and polymerization related genes.

Gene names	Gene number	Chr	Pos Mbp	Bin
4CL1 (4CL2 Lubberstedt)	GRMZM2G075333	5	89,15	5.04
4CL2 (4CL1 Lubberstedt)	GRMZM2G048522	1	210,10	1.07
ABC transporter AtABCG29-like	GRMZM2G479018	2	39,75	2.04
ABC transporter AtABCG29-like	GRMZM2G000614	3	201,54	3.07
ABC transporter ATPDR6-like	GRMZM2G391815	1	300,53	1.12
ABC transporter ATPDR6-like	GRMZM2G003411	4	231,40	4.09
ABC transporter ATPDR6-like	GRMZM2G319138	10	99,21	10.04
ABC transporter ATPDR7-like	GRMZM2G118243	3	10,27	3.03
ABC transporter EucaWood ctg6414/ PGP13	GRMZM2G085111	8	0,40	8,00
ABC transporter MaizeWall 3024030.2.1	GRMZM2G135199	1	299,98	1.12
ABC transporter MaizeWall 3871923.2.1	GRMZM2G177812	2	23,88	2.03
C3H1	GRMZM2G138074	3	187,71	3.06
C3H2	GRMZM2G140817	6	155,65	6.06
C4H1	GRMZM2G139874	8	83,79	8.03
C4H2	GRMZM2G010468	8	170,45	8.08
CAD ZmCAD1 (EgCAD1 like)	GRMZM2G179981	5	130,66	5.04
CAD ZmCAD2 (bm1, EgCAD2 like)	GRMZM5G844562	5	99,00	5.04
CAD10 (EgCAD2-like) AtCAD3/6/9	GRMZM2G700188	7	108,34	7.02
CAD6/SAD (EgCAD2 like) AtCAD3/6/9	GRMZM2G046070	2	10,53	2.02
CAD9 (EgCAD2-like) AtCAD3/6/9	AC234163.1_FG002	7	108,25	7.02
CCoAOMT1	GRMZM2G127948	6	79,19	6.01
CCoAOMT2	GRMZM2G099363	9	16,32	9.02
CCoAOMT3	GRMZM2G004138	2	189,28	2.07
CCoAOMT4	GRMZM2G033952	4	198,08	4.08
CCoAOMT5	GRMZM2G332522	4	198,08	4.08
CCR1 (ZmCCR1)	GRMZM2G131205	1	211,52	1.07
CCR2 (ZmCCR2)	GRMZM2G131836	7	47,72	7.02
CCR8 AtCCR2-like	GRMZM2G099420	4	171,78	4.06
CCR-like	GRMZM2G050076	7	133,61	7.03
COMT (bm3)	AC196475.3_FG004	4	32,25	4.04
F5H1 (Cytochrome P450 CYP84A33v1)	AC210173.4_FG005	1	224,04	1.07
F5H2	GRMZM2G100158	5	22,66	5.03
HCT1	GRMZM2G035584	5	183,61	5.05
HCT2	GRMZM2G158083	2	31,83	2.04
OMT ZRP4-like1	GRMZM2G408458	4	18,37	4.03
OMT ZRP4-like2a	GRMZM2G036048	2	126,76	2.05
OMT ZRP4-like2b SBP1	GRMZM2G085924	2	127,09	2.05
OMT ZRP4-like2c	GRMZM2G147491	2	118,54	2.05
OMT ZRP4-like3a	GRMZM2G093092	9	119,78	9.04
OMT ZRP4-like3b	GRMZM2G106172	9	119,84	9.04
OMT ZRP4-like4	GRMZM2G097297	4	192,92	4.08
OMT ZRP4-like5a	GRMZM2G140996	6	158,66	6.06
OMT ZRP4-like5b	GRMZM2G141026	6	158,69	6.06
OMT ZRP4-like5c	GRMZM2G102863	6	158,76	6.06
OMT ZRP4-like5d	GRMZM2G124799	6	158,84	6.06
PAL more distant	GRMZM2G153871	4	231,70	4.09
PAL pal3-like	GRMZM2G063917	4	143,51	4.06
PAL pal3-like	GRMZM2G334660	5	186,73	5.05
PAL pal3-like	GRMZM2G170692	5	186,80	5.05
PAL2a (pal2 locus)	GRMZM2G441347	2	28,12	2.03
PAL2b (pal2 locus)	GRMZM2G118345	2	28,05	2.03
PAL3a (pal3 locus ZmPAL)	GRMZM2G074604	5	186,68	5.05
PAL3b (pal3 locus)	GRMZM2G029048	5	186,73	5.05
PAL3c (pal3 locus)	GRMZM2G081582	4	143,47	4.06
PAL3d (pal3 locus)	GRMZM2G160541	4	143,38	4.06
ZmLac Atlac2-Atlac17-like	GRMZM2G305526	1	39,93	1.03
ZmLac putative	GRMZM2G166857	2	229,90	2.09
ZmLac putative	GRMZM2G169033	4	181,53	4.07
ZmLac putative	GRMZM2G320786	5	207,89	5.07
ZmLac1a Atl17-like(orth1)	GRMZM2G072780	3	180,68	3.06
ZmLac1a' Atlac17-Atlac2-like	GRMZM2G164467	1	260,07	1.09
ZmLac1a Atlac2-Atlac17-like	GRMZM2G447271	8	168,76	8.07
ZmLac2 (multicopper oxidase 3)	GRMZM2G141376	9	77,55	9.03
ZmLac3 Atlac12-like	GRMZM2G388587	4	183,73	4.08
ZmLac4 Atlac12-like	GRMZM5G814718	1	46,41	1.03
ZmLac5 lac1	GRMZM5G842071	3	179,12	3.06
ZmLac6 Atlac2/12/17-like	GRMZM2G146152	6	151,54	6.05

Gene names	Gene number	Chr	Pos Mbp	Bin
ZmLac7 Atlac12-like(orth1)	GRMZM2G132169	3	183,65	3.06
ZmLac8 Atlac12-like	GRMZM2G336337	8	170,04	8.08
ZmPox12	GRMZM2G103342	3	146,52	3.05
ZmPox2	GRMZM2G040638	1	63,65	1.04
ZmPox3	GRMZM2G135108	6	125,01	6.05
ZmPox39	GRMZM2G085967	5	47,58	5.03
ZmPox54	GRMZM2G088765	1	110,49	1.05

Supplementary table 5 - List of putative maize candidate genes involved in cell wall biosynthesis and assembly. Genes of the the S-adenosyl-l-methionine cycles.

Gene names	Gene number	Chr	Pos Mbp	Bin
Homocysteine S-methyltransferase HMT1	AC233893.1_FG001	9	146,26	9.06
Homocysteine S-methyltransferase HMT2	GRMZM2G117240	1	171,88	1.05
Homocysteine S-methyltransferase HMT3	GRMZM2G152470	3	121,07	3.04
Homocysteine S-methyltransferase HMT4	GRMZM2G039166	3	192,22	3.06
Methionine synthase (At3g03780, AtMS2)	GRMZM2G165747	1	259,21	1.09
Methionine synthase (At5g17920, AtMS1)	GRMZM2G149751	1	176,84	1.05
Methionine synthase (AtMS1, AtMS2)	GRMZM2G112149	5	15,55	5.02
Methylenetetrahydrofolate reductase (At2g44160, MTHFR2)	GRMZM2G034278	5	2,86	5.00
Methylenetetrahydrofolate reductase (At3g59970, MTHFR1) (bm2)	GRMZM2G347056	1	292,09	1.12
S-adenosyl-homocysteine hydrolase (At3g23810, SAHH2)	GRMZM2G111909	2	120,93	2.05
S-adenosyl-homocysteine hydrolase (At4g13940, SAHH1)	GRMZM2G015295	4	21,14	4.03
S-adenosylmethionine synthetase SAMS1	AC199526.4_FG001	3	58,90	3.04
S-adenosylmethionine synthetase SAMS2	GRMZM2G054123	8	38,41	8.03
S-adenosylmethionine synthetase SAMS3	GRMZM2G117198	8	129,62	8.05
S-adenosylmethionine synthetase SAMS4	GRMZM2G061135	10	86,41	10.03
S-AdoMet Methionine S-methyltransferase (At5g49810, MMT)	GRMZM2G098039	8	135,05	8.05

Supplementary table 6 - List of putative maize candidate genes involved in cell wall biosynthesis and assembly. Genes involved in arabinoxylan feruloylation.

Gene names	Gene number	Chr	Pos Mbp	Bin
Transferase Ara-CoA-acylT pCA-transferase (PF02458)	GRMZM2G305900	3	9,85	3.03
Transferase Ara-CoA-acylT pCA-transferase (PF02458)	GRMZM2G108714	6	128,05	6.05
Transferase Ara-CoA-acylT pCA-transferase (PF02458)	GRMZM2G375159	8	18,44	8.03
Transferase Ara-CoA-acylT pCA-transferase (PF02458)	GRMZM2G060210	8	90,58	8.03
Transferase Ara-CoA-acylT pCA-transferase (PF02458)	GRMZM2G159641	9	87,36	9.03
Transferase Ara-CoA-acylT1 (PF02458)	GRMZM2G314898	3	8,30	3.03
Transferase Ara-CoA-acylT1 (PF02458)	GRMZM2G107027	6	103,32	6.03
Transferase Ara-CoA-acylT2 (PF02458)	GRMZM2G094428	3	225,34	3.09
Transferase Ara-CoA-acylT2 (PF02458)	GRMZM2G050072	8	143,28	8.05
Transferase Ara-CoA-acylT2 (PF02458)	GRMZM2G050270	8	143,28	8.05
Transferase BAHD pCA-CoA acyltransferase	GRMZM2G130728	6	17,95	6.00
Transferase BAHD pCA-CoA acyltransferase	GRMZM2G028104	10	86,98	10.03
ZmALDH RF2C	GRMZM2G097699	3	221,71	3.09
ZmALDH RF2D	GRMZM2G071021	3	221,69	3.09

Supplementary table 7 - List of putative maize candidate genes involved in cell wall biosynthesis and assembly. Transcription factors of the MYB and NAC families, and related genes.

Gene names	Gene number	Chr	Pos Mbp	Bin
Basic helix-loop-helix bHLH	GRMZM2G017586	4	35,58	4.05
Basic helix-loop-helix bHLH	GRMZM2G082586	7	128,71	7.03
Basic helix-loop-helix bHLH	GRMZM2G089501	7	130,24	7.03
Basic helix-loop-helix bHLH	GRMZM2G049229	9	112,01	9.04
Basic helix-loop-helix bHLH	GRMZM2G014282	10	4,90	10.01
Basic helix-loop-helix bHLH	GRMZM2G093744	10	83,59	10.03
Basic helix-loop-helix bHLH	GRMZM2G009478	2	188,53	2.07
Basic-leucine zipper (bZIP)	GRMZM2G157722	4	57,68	4.05
Basic-leucine zipper (bZIP)	GRMZM2G133331	6	116,75	6.04
Basic-leucine zipper (bZIP) ABF2	GRMZM2G478417	1	199,49	1.07
KNAT7-like1 At1g62990 (ELK KNOX1)	GRMZM2G159431	1	5,01	1.01
KNAT7-like2 At1g62990 (ELK KNOX1)	GRMZM2G055243	9	92,87	9.03
KNAT7-like3 (ELK KNOX1)	GRMZM2G060507	9	153,74	9.07
KNAT7-like4 (ELK KNOX1)	GRMZM2G433591	4	235,21	4.09
KNAT7-like5 (ELK KNOX1)	GRMZM2G370332	5	94,45	5.04
MYB (G13) ZmMYB019 AtMYB61-like	GRMZM2G147698	1	64,24	1.04
MYB (G13) ZmMYB044 AtMYB61-like	GRMZM2G052377	3	13,2	3.03
MYB (G13) ZmMYB045 AtMYB61-like	GRMZM2G064744	3	227,39	3.09
MYB (G13) ZmMYB100 AtMYB61-like	GRMZM2G175232	6	162,11	6.07
MYB (G13) ZmMYB117 AtMYB61-like	GRMZM2G003406	8	153,46	8.05
MYB (G13) ZmMYB123 AtMYB61-like	GRMZM2G119693	8	63,55	8.03
MYB (G13) ZmMYB126 AtMYB61-like	GRMZM2G171781	8	129,07	8.05
MYB (G13) ZmMYB150 AtMYB61-like	GRMZM2G127490	10	87,79	10.03
MYB (G13) ZmMYB040 AtMYB61-like	GRMZM2G017520	3	48,49	3.04
MYB (G13) ZmMYB114 AtMYB61-like	GRMZM2G150841	7	168,29	7.05
MYB (G2) ZmMYB027 AtMYB58-like	GRMZM2G048295	2	29,54	2.03
MYB (G2) ZmMYB069 AtMYB58-like	GRMZM2G127857	4	142,64	4.06
MYB (G2) ZmMYB083 AtMYB58-like	GRMZM2G095904	5	186,41	5.05
MYB (G2) ZmMYB144 AtMYB58-like	AC206901.3_FG005	10	130,72	10.05
MYB (G28) ZmMYB130 AtMYB5-like	GRMZM2G395672	8	152,77	8.05
MYB (G29) ZmMYB047 AtMYB61-like	GRMZM2G088783	3	204,15	3.07
MYB (G29) ZmMYB116 AtMYB61-like	GRMZM2G172327	7	150,09	7.03
MYB (G3) ZmMYB026 AtMYB63-like	GRMZM2G038722	2	13,30	2.02
MYB (G3) ZmMYB075 AtMYB63-like	GRMZM5G833253	4	157,77	4.06
MYB (G3) ZmMYB148 AtMYB63-like	GRMZM2G097636	10	140,19	10.07
MYB (G3) ZmMYB149 AtMYB63-like	GRMZM2G097638	10	140,19	10.07
MYB (G31) ZmMYB146 (MYB46) EgMYB2-like	GRMZM2G052606	10	28,60	10.03
MYB (G4) ZmMYB010 AtMYB4-like	GRMZM2G084583	1	206,86	1.07
MYB (G4) ZmMYB028 (MYB31) AtMYB4-like	GRMZM2G050305	2	196,29	2.07
MYB (G4) ZmMYB035 AtMYB4-like	GRMZM2G124715	2	131,35	2.05
MYB (G4) ZmMYB041 AtMYB4-like	GRMZM2G041415	3	140,12	3.05
MYB (G4) ZmMYB052 AtMYB4-like	GRMZM2G160838	3	173,52	3.06
MYB (G4) ZmMYB065 AtMYB4-like	GRMZM2G089244	4	206,87	4.09
MYB (G4) ZmMYB073 (MYB42) AtMYB4-like	GRMZM2G419239	4	216,61	4.09
MYB (G4) ZmMYB094 AtMYB4-like	GRMZM2G077789	6	150,69	6.05
MYB (G4) ZmMYB106 AtMYB4-like	GRMZM2G000818	7	176,13	7.05
MYB (G4) ZmMYB131 AtMYB4-like	GRMZM2G405094	8	165,88	8.07
MYB (G4) ZmMYB132 AtMYB4-like	GRMZM2G431156	8	101,78	8.03
MYB (G8) ZmMYB012 AtMYB85-like	GRMZM2G037650	1	207,18	1.07
MYB (G8) ZmMYB064 AtMYB85-like	GRMZM2G055158	4	166,91	4.06
MYB (G8) ZmMYB071 AtMYB85-like	GRMZM2G138427	4	216,21	4.09
MYB (G8) ZmMYB003 AtMYB85-like	GRMZM2G106558	1	215,34	1.07
MYB (G8) ZmMYB003 AtMYB85-like	GRMZM2G104551	7	143,21	7.03
MYB (G8) ZmMYB113 AtMYB85-like	GRMZM2G126566	7	108,79	7.02
MYB (G8) ZmMYB115 AtMYB85-like	GRMZM2G169356	7	108,70	7.02
MYB (G8) ZmMYB92 AtMYB85-like	GRMZM2G048910	6	117,95	6.04
MYB (G30) ZmMYB156 AtMYB103-like	GRMZM2G325907	10	81,83	10.03
MYB (G21) ZmMYB059 AtMYB69-like	GRMZM5G803355	3	138,4	3.05
MYB (G21) ZmMYB090 AtMYB52-like	GRMZM2G455869	5	10,17	5.02
MYB (G21) ZmMYB008 AtMYB54-like	GRMZM2G077147	1	269,64	1.09
NAC ZmNAC AtNAC082 VNI1 At5g09330	GRMZM2G340305	1	203,09	1.07
NAC ZmNAC AtNAC082 VNI1 At5g09330	GRMZM2G176677	2	26,43	2.03
NAC ZmNAC AtNAC082 VNI1 At5g09330	GRMZM2G125777	4	50,10	4.05
NAC ZmNAC AtNAC082 VNI1 At5g09330	GRMZM2G456568	6	147,92	6.05
NAC ZmNAC AtNAC082 VNI1 At5g09330	GRMZM2G104400	8	102,54	8.03
NAC ZmNAC AtNAC083 VNI2 At5g13180	GRMZM2G123667	4	207,98	4.09
NAC ZmNAC AtNAC083 VNI2 At5g13180	GRMZM2G336533	5	2,89	5.00

Gene names	Gene number	Chr	Pos Mbp	Bin
NAC ZmNAC AtNAC083 VNI2 At5g13180	GRMZM2G179885	7	158,24	7.04
NAC ZmNAC AtNAC083 VNI2 At5g13180	GRMZM2G068973	8	170,86	8.08
NAC ZmNAC AtNAC083 VNI2 At5g13180	GRMZM2G126936	9	145,84	9.06
NAC ZmNAC AtNAC083 VNI2 At5g13180	GRMZM2G430849	7	173,52	7.05
NAC ZmNAC AtXND1-like ZmNAC037/079	GRMZM2G094067	5	172,92	5.05
NAC ZmNAC AtXND1-like ZmNAC096	GRMZM2G316840	2	48,82	2.04
NAC ZmNAC NST1-like ZmNAC076 ZmSWN7a	GRMZM2G041668	9	28,04	9.03
NAC ZmNAC NST1-like ZmNAC169 ZmSWN6	GRMZM2G178998	2	22,70	2.03
NAC ZmNAC NST1-like ZmNAC176 ZmSWN7b	GRMZM2G440219	9	28,14	9.03
NAC ZmNAC SND1/NST1-like	GRMZM2G155816	5	142,18	5.04
NAC ZmNAC SND1/NST1-like	GRMZM2G104074	9	64,93	9.03
NAC ZmNAC SND1/NST1-like ZmNAC014	GRMZM2G092465	6	4,31	6,00
NAC ZmNAC SND1/NST1-like ZmNAC143 ZmSWN2	GRMZM2G069047	4	38,03	4.05
NAC ZmNAC SND1-like	GRMZM2G435824	10	119,13	10.04
NAC ZmNAC SND1-like ZmNAC020	GRMZM2G091490	6	66,03	6.01
NAC ZmNAC SND1-like ZmNAC091	GRMZM2G099144	2	47,05	2.04
NAC ZmNAC SND1-like ZmNAC5	GRMZM2G041746	6	106,25	6.03
NAC ZmNAC SND2/SND3 ANAC010	GRMZM2G166721	3	6,94	3.02
NAC ZmNAC SND2/SND3 ARGOS ZmNAC080/156	GRMZM2G137546	2	220,00	2.08
NAC ZmNAC SND2/SND3-like	GRMZM2G112681	8	20,83	8.03
NAC ZmNAC SND2/SND3-like ZmNAC101/182	GRMZM2G058518	3	210,23	3.08
NAC ZmNAC SND2/SND3-like ZmNAC123	GRMZM2G031200	6	164,50	6.07
NAC ZmNAC SND3/SND2-like ZmNAC019	GRMZM2G134717	8	149,81	8.05
NAC ZmNAC VND1 ZmNAC184 ZmSWN5	GRMZM2G025642	1	5,64	1.01
NAC ZmNAC VND6-like	GRMZM2G172053	9	153,53	9.07
NAC ZmNAC VND6-like ANAC012	GRMZM2G315140	5	188,92	5.05
NAC ZmNAC VND6-like ZmNAC178	GRMZM2G354151	4	148,82	4.06
NAC ZmNAC VND7-like ZmNAC168/186 ZmSWN4	AC212859.3_FG008	2	25,75	2.03
NAC ZmNAC VND7-like ZmNAC032/118 ZmSWN1	GRMZM2G171395	9	23,28	9.03
NAC ZmNAC VND7-like ZmNAC076 ZmSWN3	GRMZM2G052239	6	0,78	6,00
NAC ZmNAC VND7-like ZmNAC115	GRMZM2G048826	4	59,34	4.05
Ovate family protein OFP1/4/17-like	GRMZM2G026927	6	153,84	6.06
Ovate family protein OFP1/4-like	GRMZM2G075988	1	56,31	1.03
Ovate family protein OFP1/4-like	GRMZM2G330159	3	184,31	3.06
Ovate family protein OFP1/4-like	GRMZM2G127680	6	159,01	6.06
Ovate family protein OFP1/4-like	GRMZM2G067376	8	91,86	8.03
Ovate family protein OFP1/4-like	AC204502.4_FGP006	8	170,28	8.08
Ovate family protein OFP4-like	GRMZM2G127431	3	198,04	3.07
Ovate family protein OFP4-like	GRMZM5G845472	7	172,97	7.05
Ovate family protein OFP4-like	GRMZM2G312221	8	109,95	8.03
WD40 repeat-like EW CCAAT-HAP5	GRMZM2G038032	6	162,88	6.07
WD40 repeat-like EW CCAAT-HAP5	GRMZM2G040477	8	65,75	8.03
WD40 repeat-like EW WD40	GRMZM2G022627	2	36,94	2.04
WD40-like (EgMYB2 Prt)	GRMZM2G123709	9	126,00	9.04

Supplementary table 8 - List of putative maize candidate genes involved in cell wall biosynthesis and assembly. Miscellaneous genes involved in regulation of lignified tissue assembly.

Gene names	Gene number	Chr	Pos Mbp	Bin
AtMAP70-1 At1g68060	GRMZM5G832989	5	205,31	5.06
AtMAP70-1 At1g68060	GRMZM2G008556	6	115,64	6.04
AtMAP70-1 At1g68060	GRMZM2G039325	9	0,57	9,00
COV LCV2-like	GRMZM2G149662	3	175,56	3.06
COV LCV2-like	GRMZM2G048150	5	20,68	5.03
COV LCV2-like	GRMZM2G073415	6	150,26	6.05
COV LCV2-like	GRMZM2G052855	8	100,96	8.03
COV LCV2-like	GRMZM2G046098	8	166,41	8.07
COV LCV3-like	GRMZM2G125985	10	13,55	10.02
COV1-like	GRMZM2G123790	4	222,80	4.09
COV1-like	GRMZM2G146511	5	136,77	5.04
COV1-like	GRMZM2G101533	6	159,77	6.06
COV1-like	GRMZM2G052200	8	125,01	8.05
GRAS SCARECROW-like1 At1g21450	GRMZM2G431309	2	207,84	2.08
GRAS SCARECROW-like1 At1g21450	GRMZM5G885274	5	205,08	5.06
GRAS SCARECROW-like1 At1g21450	GRMZM2G153333	6	147,91	6.05
GRAS SCARECROW-like1 At1g21450	GRMZM2G098517	7	161,29	7.04
GRAS SCARECROW-like1 At1g21450	GRMZM2G028039	9	149,17	9.07
HD-ZIP ATHB8 HD-ZIPIII (PtrHD7-like)	GRMZM2G469551	1	230,54	1.07
HD-ZIP ATHB-8 HD-ZIPIII (PtrHD7-like)	GRMZM2G178102	3	123,27	3.04
HD-ZIP ATHB-8 HD-ZIPIII bZIP (PtrHD7-like)	GRMZM2G003509	1	173,23	1.05
HD-ZIP IFL1 (ATHB8) HD-ZIPIII rld1 (PtrHD7-like)	GRMZM2G109987	9	154,65	9.07
HD-ZIP IFL1 HD-ZIPIII rld2 (ATHB8) bZIP (PtrHD7-like)	GRMZM2G042250	1	2,80	1.01
HD-ZIP ATHB-8 HD-ZIPIII (PtrHD7-like)	AC187157.4_FG005	8	22,64	8.03
ROP AtROP3 EgROP1 At2g17800	GRMZM2G001953	4	182,19	4.07
ROP AtROP3 EgROP1 At2g17800	AC209819.3_FG012	8	122,95	8.05
ROP AtROP3 EgROP1 At2g17800	GRMZM2G102946	9	3,07	9,00
ROP family GTPase ROP2 AtROP3 EgROP1	GRMZM5G846811	4	238,05	4.10
ROP family GTPase ROP9 AtROP3 EgROP1	GRMZM5G803949	5	70,88	5.03
ROP Ras small GTPase Rho type AtROP3 EgROP1	GRMZM2G375002	5	217,60	5.09
ROP Ras small GTPase Rho type AtROP3 EgROP1	GRMZM2G415327	5	206,15	5.06
ROP Ras small GTPase Rho type EU968843.1*	GRMZM2G073609	5	217,61	5.09
ROP Ras small GTPase ROP6 AtROP3 EgROP1	GRMZM2G176217	6	158,45	6.06
SHINE1/ SHINE2/ SHINE3	GRMZM2G085678	5	114,10	5.04
SHINE1/ SHINE2/ SHINE3/ AP2/ERF	GRMZM2G106591	6	102,42	6.03
SHP1 MADSbox-like AGL11 STK (SEEDSTICK)	GRMZM2G052890	6	131,83	6.05
SHP1 MADSbox-like Kbox AP1 (APETALA1)	GRMZM2G072582	7	2,07	7,00
SHP1 MADSbox-like MADSbox	GRMZM2G160687	3	137,23	3.05
SHP1 MADSbox-like MADSbox	GRMZM2G160565	5	196,55	5.06
SHP1 MADSbox-like MADSbox	GRMZM2G359952	8	22,98	8.03
SHP1 MADSbox-like MADSbox	GRMZM2G010669	10	30,87	10.03
SHP1 ZmZAG5 Shatterproof Agamous	GRMZM2G003514	4	156,09	4.06
Zinc finger C2H2-like	GRMZM2G048154	1	42,12	1.03
Zinc finger C2H2-like	GRMZM2G112251	9	139,17	9.05
Zinc finger C2H2-like	GRMZM2G165355	9	143,86	9.06
Zinc finger C2H2-like	GRMZM2G095323	10	37,64	10.03
Zinc finger C2H2-like indeterminate growth1 (id1)	GRMZM2G171073	1	23,57	1.02
Zinc finger C2H2-like lot of est	GRMZM5G887286	2	222,16	2.08
Zinc finger C2H2-like lot of est	GRMZM5G898314	8	163,12	8.06
Zinc finger C2H2-like RNA recognit motif RNP-1	GRMZM2G019266	3	172,63	3.06
Zinc finger C2H2-like RNA recognit motif RNP-1	GRMZM2G005236	8	165,55	8.06
Zinc finger C3HC4-type RING	GRMZM2G077307	3	201,99	3.07
Zinc finger C3HC4-type RING	GRMZM2G056270	10	25,30	10.03
Zinc finger C3HC4-type RING lot EST	GRMZM2G062724	1	10,04	1.01
Zinc finger CCCH-type AtC3H14-like	GRMZM5G830949	8	146,98	8.05
Zinc finger CCCH-type AtC3H14-like1	GRMZM2G157927	3	214,75	3.09
Zinc finger CCCH-type AtC3H14-like1	GRMZM2G149347	6	165,86	6.07
Zinc finger CCCH-type AtU2AF35b	GRMZM2G025014	2	232,64	2.09
Zinc finger CCCH-type AtU2AF35b	GRMZM2G020928	6	164,57	6.07
Zinc finger CCCH-type AtU2AF35b	GRMZM2G177229	7	133,53	7.03
Zinc finger CCCH-type AtU2AF35b	GRMZM2G031827	8	70,14	8.03
Zinc finger CCCH-type C3H	GRMZM2G148090	9	155,98	9.07
Zinc finger CCCH-type RNA recognit motif RNP-1	GRMZM2G168163	2	186,90	2.07
Zinc finger CCCH-type RNA recognit motif RNP-1	GRMZM2G467907	7	127,15	7.02
Zinc finger DOF-typa HCA2 At5g62940	GRMZM2G589696	4	160,28	4.06
Zinc finger DOF-typa HCA2 At5g62940	GRMZM2G140694	5	201,38	5.06
Zinc finger DOF-type At1g21340	GRMZM2G135703	3	175,97	3.06

Gene names	Gene number	Chr	Pos Mbp	Bin
Zinc finger DOF-type At1g21340	GRMZM2G371058	6	149,13	6.05
Zinc finger DOF-type At1g21340	GRMZM2G042218	8	166,58	8.07
Zinc finger DOF-type At1g64620	GRMZM2G010290	10	137,21	10.06
Zinc finger DOF-type At3g21270	GRMZM2G011832	1	246,48	1.08
Zinc finger DOF-type At3g21270	GRMZM2G084130	5	6,11	5.01
Zinc finger DOF-type At3g21270	GRMZM2G178767	5	19,53	5.03
Zinc finger Lim-type	GRMZM2G024887	10	132,70	10.05
Zinc finger Lim-type Ntlim1/2-like	GRMZM2G175761	3	134,61	3.05
Zinc finger Lim-type Ntlim1-like	GRMZM2G485184	6	21,97	6.01
Zinc finger Lim-type ZmLim3 MaizeWall	GRMZM2G153268	2	23,13	2.03
Zinc finger WRKY ZmWRKY15 AtWRKY12-like	GRMZM2G123387	2	21,05	2.02
Zinc finger WRKY ZmWRKY50 AtWRKY12-like	GRMZM2G377217	4	151,07	4.06
Zinc finger WRKY ZmWRKY110 VvWRKY2-like	GRMZM2G171428	9	124,23	9.04
Zinc finger WRKY ZmWRKY23 VvWRKY2-like	GRMZM2G130854	2	207,84	2.08
Zinc finger WRKY ZmWRKY82 VvWRKY2-like	GRMZM2G398506	7	161,29	7.04
Fasciclin AtFLA11/IRX13-like AtFLA12-like	GRMZM2G022931	3	210,51	3.08
Fasciclin AtFLA11/IRX13-like AtFLA12-like	GRMZM2G177242	3	15,06	3.03
Fasciclin AtFLA4-like (SOS5 mutant)	AC213621.5_FGT004	6	151,58	6.05
Fasciclin AtFLA4-like (SOS5 mutant)	GRMZM2G421415	8	116,49	8.04
Fasciclin AtFLA4-like (SOS5 mutant)	GRMZM2G035933	8	168,94	8.07