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Comparative expression of cell wall related genes in four maize RILs and one parental line of variable lignin content and cell wall degradability

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Abstract

A comparison of gene expression in maize between the parental line F271 and four RILs derived from the cross F288 x F271 was investigated based on hybridization on the 17,555 probes Affymetrix micro-array, targeting nearly one third of the genes present in maize genomes. The parental line had unfavorable alleles for cell wall degradability traits at the major QTL position in bin 6.06, while the set of RILs had both the favorable allele and high cell wall degradability. 360 genes were differentially expressed in the four RIL in comparison to F271, including nine genes underlying the major QTL position and 36 underlying two other QTL positions. However, their proposed function (whenever is described) do not allow us to firmly consider their involvement in the observed variation of cell wall related traits. Only a few genes involved in monolignol biosynthesis or polymerization located elsewhere in the genome were differentially expressed between the four RILs and F271, corroborating with the fact that these genes are probably not involved in major determinants of cell wall degradability in the studied set of lines. Among the investigated regulation factors, three *ZmMYB*, one *NAC* and one C3HC4 zinc finger were differentially expressed between the four RILs and F271, but they were not located in bin 6.06. Notwithstanding, the obtained results especially strengthened the probable involvement of these genes in maize secondary wall assembly and/or lignification.

Keywords: maize, degradability, lignin, cell wall, expression

Introduction

Most plant species dedicated to ruminant nutrition and/or proposed as feedstock for renewable energy production belong to the grass clade. Even if a variable part of soluble carbohydrates is still present in harvested grasses, most plant biomass is made up of secondary cell walls which are mostly comprised of cellulose and hemicelluloses embedded in a phenolic component matrix. This matrix is constituted of lignins and *p*-hydroxycinnamic acids which contribute to the plant standability and confer hydrophobicity to the vascular elements allowing transport of water and nutriments. Constitutive and neo-synthesized stress-lignins are involved in defense responses against diseases and pests. On the other hand, lignification is also the basic cause of reduced digestibility of forages in animal digestive tracks and of reduced conversion of plant biomass into bioethanol. Because of the negative impact of lignins for the pulp and paper industry, a sector of considerable economic importance, most of the efforts in genetics, genomics, and biochemistry have been directed towards a better understanding of lignin biosynthesis in perennial and annual dicotyledonous including the Arabidopsis model system as well as in gymnosperm species. Conversely, fewer studies have been carried on in grasses. Deciphering the genetic determinants of cell wall biosynthesis and assembly in grasses is, thus, a strategic issue in breeding plants for both cattle feeding and second generation biofuel production.

Due to the large genetic resources characterized for cell wall related traits, the ease of self-pollination and crossing, the availability of genomic tools (ie. EST, transgenesis, transposon-tagging), and the recent release of its genomic sequence (Schnable et al, 2009), maize is both a plant of direct high economical interest and a model system for all grasses. QTL analyses for lignin and cell wall related traits have been investigated in several maize RIL progenies (Méchin et al, 2001; Roussel et al, 2002; Krakowsky et al, 2003; Cardinal et al, 2003; Krakowsky et al, 2005; Barrière et al, 2008; Riboulet et al, 2008a; Barrière et al, 2010;

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Barrière et al, 2012). Even if co-localizations between candidate genes and QTLs have been pointed out in maize, but also in arabidopsis, poplar, and eucalyptus (Barrière et al, 2010; Ranjan et al, 2010; Thomas et al, 2010; Gion et al, 2011; Chavigneau et al, 2012), no gene has yet been shown to be responsible for the effect of any cell wall-related QTL. The latter situation is due in part to our insufficient knowledge of the major genetic determinants of cell wall biosynthesis, assembly and regulation, even if mutants, including brown-midrib mutants in maize, and genetically modified plants in the lignin pathway have been studied, and candidate genes listed in both maize and Arabidopsis (He et al, 2003; Barrière et al, 2004; Ko and Han, 2004; Minic et al, 2009; Pichon et al, 2006; Barrière et al, 2009; Tamasloukht et al, 2011; Chavigneau et al, 2012). The size of the QTL support intervals, which have an average length of nearly 20 cM corresponding in maize to 15 to 50 Mbp, and up to 500 genes, depending on their genomic location and recombination rate, is another reason for making candidate gene identification and validation difficult.

In the F288 x F271 early maize RIL progeny, several QTLs for cell wall related traits, including lignin content and cell wall degradability, were already mapped (Roussel et al, 2002; Thomas et al, 2010). Among the latter QTLs, those located in bin 6.06 explained a highly significant part of investigated trait variations, with R^2 values ranging from 20 to 39% for investigated traits. In order to contribute to the identification of candidate genes underlying QTLs, a large-scale gene expression study was carried out, comparing the parental line (F271) with unfavorable alleles for cell wall degradability traits in bin 6.06 and a set of four RILs with both favorable phenotypic and allelic values. Because the objective of the expression study was to get insights into the discovery of genes involved in cell wall variation for degradability and lignin content, mostly in relation with QTLs in bin 6.06, two strategies of gene expression study were simultaneously considered. Gene expressions between the F271 parental line and the set of RILs were investigated with a targeted hypothesis-driven strategy, considering genes putatively involved in cell wall biosynthesis and assembly, and regulation of these processes according to the list proposed by Barrière et al (2009). In addition, a screen for genes giving a significant differential expression between F271 and each of the RILs was considered, first focusing on those located in bin 6.06 QTL support intervals, and then on those elsewhere with a genome-wise approach.

Materials and Methods

RIL progeny development and experiments

A set of 131 RILs was initially developed by single seed descent from the cross between the two early dent inbred lines F288 and F271, which are of medium-high and low cell wall degradability, respectively (Barrière et al, 2001). RIL progenies were then evaluated in field experiments for both their per se and topcross values, with F286 as flint tester of high cell wall degradability (Roussel et al, 2002). QTLs were detected for cell wall related traits, including lignin content (ADL/NDF and KL/NDF) and cell wall digestibility (IVNDFD and DINAGZ). NDF (Neutral Detergent Fiber) is an estimate of cell wall content and ADL (Acid Detergent Lignin) is an estimate of lignin content according to Goering and van Soest (1970). KL (Klason lignin) is gravimetric method used to assess the lignin content according to Dence and Lin (1992) including the acido-soluble part of lignin which is lost during the first step of the ADL procedure (Hatfield et al, 1994; Jung et al, 1997; Hatfield and Fukushima, 2005). IVNDFD (in vitro NDF digestibility) is an estimate of cell wall digestibility, based on the enzymatic solubility of Aufrère and Michalet-Doreau (1983), which is calculated according to Struik (1983) and Dolstra and Medema (1990), assuming that the non-NDF part is fully digestible. DINAGZ is another estimate of cell wall digestibility, similarly based on the same enzymatic solubility, which is estimated according to Argillier et al (1995) and Barrière et al (2003), assuming that starch, crude proteins, and soluble carbohydrates are fully digestible. Major QTLs were, thus, shown for these four cell wall traits in bin 6.06 (Table 1).

Line choice

The parental line F271, with alleles giving high lignin content and low cell wall degradability values at all QTL positions except in bin 9.02, was chosen as the "negative" control. Four RILs (RIL39, RIL54, RIL99, and RIL118) were chosen, firstly, for their parental F288 genotypes in bin 6.06, based on three markers, from positions 147.0 to 166.3 Mbp which overlapped QTL support intervals (Figure 1). The RIL39 differed from the three other RILs because it has F271 parental allele downstream QTL positions, while RIL99, RIL54, and RIL118 have F288 allele up until phi089 marker (position 166.3 Mbp). RILs were also chosen for their phenotypic values with high cell wall degradability and low lignin contents (Table 2). The four RILs had also the favorable alleles (increasing cell wall degradability) at QTL positions in bins

Table 1 - Position (cM), Lod value, explained phenotypic variation (R2, %), and additive value (add) of putative QTLs identified in bin 6.06 for ADL/NDF and KL/NDF lignin content, and DINAGZ and IVNDFD cell wall digestibility [131 RIL of the F288 x F271 progeny in per se experiments, from Roussel et al (2002) and Thomas et al (2010), traits as percentage].

QTL bin 6.06	Position	Lod	R2	add value
ADL/NDF	142	6.2	19.5	0.30
KL/NDF	142	9.0	27.0	0.66
DINAGZ	142	8.4	25.6	1.26
IVNDFD	142	14.3	39.4	2.50

Figure 1 - Physical map of the genome of the four RILs (colour bars) and of the QTLs for cell wall related trait in the RIL F288 x F271 progeny. Candidate genes are positioned on the right bars (white bars). In green= F288 allele, blue= F271 allele, black= unknown allele and yellow= heterozygote.

Table 2 - Phenotypic values in the parental lines and the set of selected RILs for ADL/NDF and KL/NDF lignin content, and DINAGZ and IVNDFD cell wall digestibility [131 RIL of the F288 x F271 progeny in per se experiments, from Roussel et al (2002)].

	F288	F271	RIL39	RIL99	RIL54	RIL118
ADL/NDF	5.4	7.6	5.2	5.6	5.9	5.8
KL/NDF	13.0	16.1	12.3	12.8	13.1	13.9
DINAGZ	53.8	43.6	54.7	54.3	54.0	53.8
IVNDFD	36.9	23.3	38.4	38.4	36.7	35.2

1.02 and 3.05, while favorable alleles were not present in RIL118 in bin 9.02.

Field experiments and sampling for gene expression studies

Selected RILs and the parental line F271 were cropped at INRA Lusignan (Vienne, France) in 2009 in a block design with two four-row replicates. Row spacing was 0.75 m and the density was thinned to 90,000 plants/ha. Irrigation was applied during summer to prevent water stress.

Because Guillaumie et al (2007a, 2008) and Riboulet et al (2009) have shown that the genes of the lignin pathway were highly expressed in the below-ear internode in a period from tassel emergence (S1) to a few days after silking (S2), corresponding to the end of the elongation phase and the deposition of the secondary cell wall in this internode, below-ear internodes (without nodes) of five representative plants in each of the two replicates were, thus, harvested at this two stages S1 and S2. Sampling was done five hours after sunrise, for each of the four investigated RILs and the parental line F271. To limit experiment costs, biological repetitions of each RIL were pooled after cutting the internode in fragments of nearly 1 cm long. All samples were immediately frozen in liquid nitrogen and stored at -80°C.

RNA extraction

The internodes were crushed in liquid nitrogen using an Ika Mill crusher (IKA, Staufen, Germany) prior to RNA extraction. Total RNAs were extracted using the Nucleospin® RNA II kit (Macherey-Nagel). Some modifications of the protocol were added, including i) 600 µl of buffer RA1 were used, instead of 450 µl, and 6 µl of β-mercaptoethanol, instead of 3.5 µl, for the cell lyse, ii) 600 µl of 70% ethanol were used for the DNA binding, instead of 350 µl, and iii) finally two washes at 600 µl of buffer RA3, instead of one. RNA samples were checked for their integrity with the Agilent 2100 bioanalyzer according to the Agilent technologies (Waldbroon, Germany).

Affymetrix micro-array hybridization, statistical analysis, and gene expression investigations

One µg of total RNA was used to synthesize biotin-labeled cRNAs with the One-cycle cDNA synthesis kit (Affymetrix, Santa Clara, CA). Superscript II reverse transcriptase and T7-oligo (dT) primers were used to synthesize the single strand of cDNA at 42°C during 1 hour, followed by the synthesis of the double stranded cDNA by using DNA ligase, DNA polymerase I and RNaseH during 2 hours at 16°C. Clean up of the double-stranded cDNA was performed with Sample Cleanup Module (Affymetrix) followed by in vitro transcription (IVT) in the presence of biotin-labeled UTP using GeneChip® IVT labelling Kit (Affymetrix). Quantity of the labelled-cRNA with RiboGreen® RNA Quantification Reagent (Turner Biosystems, Sunnyvale, CA) was determined after cleanup by the Sample Cleanup Module (Affymetrix). Fragmentation of 10µg of labelled-cRNA was carried out for 35 minutes at 94°C, followed by hybridization during 16 hours at 45°C to Affymetrix GeneChip® Maize Genome Array 18K representing approximately 14,850 maize transcripts, corresponding to 13,339 genes. After hybridization, the arrays were washed with 2 different buffers (stringent: 6x SSPE, 0.01% Tween-20 and non-stringent: 100mM MES, 0.1M [Na^+], 0.01% Tween-20) and stained with a complex solution including Streptavidin R-Phycoerythrin conjugate (Invitrogen/molecular probes, Carlsbad, CA) and anti-Streptavidin biotinylated antibody (Vectors laboratories, Burlingame, CA). The washing and staining steps were performed in a GeneChip® Fluidics Station 450 (Affymetrix). The Affymetrix GeneChip® Maize Genome Arrays 18K were finally scanned with the GeneChip® Scanner 3000 7G piloted by the Command Console Launcher Tool.

The data were normalized (Log2 values) with the gcrma algorithm (Irizarry et al, 2003), available in the Bioconductor package (Gentleman et al, 2002). To determine differentially expressed genes, a usual two group t-test, that assumes equal variance between groups, was performed. The variance of the gene expression per group is a homoscedastic variance, where genes displaying extremes of variance (too small or too large) were excluded. The raw P values were adjusted by the Bonferroni method, which controls the Family Wise Error Rate (FWER, Ge et al, 2003). A gene was declared differentially expressed if the Bonferroni P-value was less than 0.05. All this steps were performed on Affymetrix platform at INRA-URGV, Evry.

The physical positions of differentially expressed genes were considered according to the B73 genomic sequence (Schnable et al, 2009, www.maizesequence.org, release v2 5b60) allowing testing of their colocalizations with QTLs, especially with QTLs in bin 6.06.

Data Deposition

The raw.CEL files were imported in R software for data analysis. All raw and normalized data are avail-

able through the CATdb database (AFFY_ZeaWall-RIL_Maize & AFFY_ZeaWall-Ril2_MAIZE, Gagnot et al, 2008) and from the Gene Expression Omnibus (GEO) repository at the National Center for Biotechnology Information (NCBI), accession number GSE22439 & GSE 27070 (Barrett et al, 2007).

Results

Global comparative analysis of gene expression between F271 and four RILs

A differential expression between F271 and all of the four RILs was only shown for 338 probes out of the 17,555 probes available on the Affymetrix microarray (Table 3); these probes corresponding to 360 genes (including probes hybridizing two close paralogs). Most of the genes differentially expressed at stage S1 were also differentially expressed at stage S2. Differentially expressed genes were classified according to the MapMan BIN classification (Thimm et al, 2004). Twenty five percent of the genes were of unknown function, while nearly 2% had a function related to cell wall metabolism, 1% belongs to the secondary metabolism functional class, and 9% had a RNA related function including regulation of transcription (Figure 2).

Comparative expression of genes related to monolignol biosynthesis and polymerization

Monolignol biosynthesis genes

Expression data were available for 22 genes belonging to nine multigene families involved in monolignol biosynthesis (Supplementary table 1A). No probes or data were available neither for F5H (ferulate-5-hydroxylase), and nor for several members in different families. Only a few differences in transcript levels were observed between the four RILs and the parental line F271. The *PAL2b* (*phenylalanine-ammonia-lyase*) gene (GRMZM2G118345) was over-expressed in all RILs at stage S2 and in three RILs out of four at stage S1 as compared to the parental line. Even if an under-expression of PAL genes should have been expected in RILs that are less lignified than F271, a higher involvement of the *PAL2b* gene could induce favorable differences in lignification patterns between the two plant types. However PAL enzymes are not specific of the lignin pathway since they catalyze the first step of the phenylpropanoid pathway which leads to many phenylpropanoids compounds other than lignins. The *C3'H1* (*cinnamate-3-hydroxylase*) gene (GRMZM2G138074) was under-expressed in RILs except RIL99, which had the F271 allele in this position (Figure 1). There was also a tendency towards an over-expression of the *CAD10* (*cinnamyl-alcohol-dehydrogenase*). This gene is a *ZmCAD2-type* gene, but its involvement in constitutive lignification has not yet been established. Finally, the *CCoAOMT3* showed a little lower expression in RIL54.

Monolignol related genes

Based on the B73 sequence (Schnable et al, 2009), 12 *ZRP4-like OMT* (*Zea Root Preferential O-methyl-transferase*) are present in the maize genome, of which its role is likely not limited to the methylation of suberin subunit precursors in plant roots as it has been initially described by Held et al (1993). It was hypothesized that member(s) of the family could be involved in methylation of caffeoyl aldehyde in a route preferentially oriented towards the biosynthesis of sinapyl alcohol (Barrière et al, 2009). The *ZRP4-like2b OMT* (GRMZM2G085924, *SBP1*, Scott-Craig et al, 1998) was over-expressed in the four RILs at stage S1 and in two RILs at stage S2, while it was under-expressed in one RIL at this stage (Supplementary table 1B). Simultaneously, a lower expression was shown, but only at stage S2, for the *ZRP4-like5c/5d OMT* gene(s), the corresponding probe targeting the two close and closely located genes GRMZM2G102863 (position 158.76 Mbp) and GRMZM2G124799 (position 158.84 Mbp). Monolignols are transported from the cytosol to the cell wall, very likely as monolignol glucosides (Lanot et al, 2006), after their glucosylation by specific uridine-diphosphate-glucosyltransferases

Table 3 - Number of probes giving significant differential expression between F271 and all of the four RILs [Expression data from below-ear internodes, at emerging tassel stage (S1) and silking stage (S2)].

	Stage S1 (only)	Stage S2 (only)	Stages S1 and S2
F271 > all 4 RIL	8	133	46
fold change ≥ 20	1	7	17
10 ≤ fold change < 20	2	16	13
5 ≤ fold change < 10	4	41	14
2 ≤ fold change < 5	1	69	2
All 4 RIL > F271	24	77	50
fold change ≥ 20	1	1	25
10 ≤ fold change < 20	6	10	11
5 ≤ fold change < 10	14	26	14
2 ≤ fold change < 5	3	40	0
Total	32	210	96

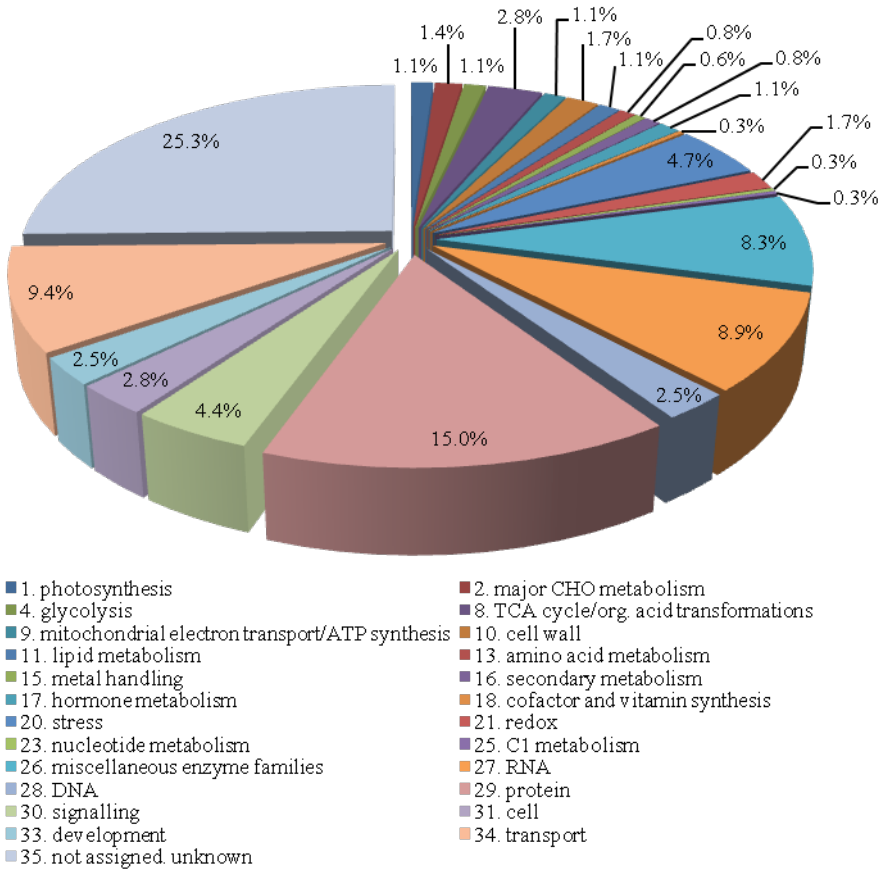


Figure 2 - Functional classification of genes differentially expressed between F271 and the four RILs (classification according to MapMan BIN, percentage of gene in each class).

(UGT, no available probes). The release of monolignol aglycone from its glucosidic form at the cell wall for subsequent lignin polymerization is thought to be mediated by specific glucosidases, such as the two Arabidopsis β-Glu45 and β-Glu46 (β-glucosidases), of which encoding genes are strongly expressed in lignifying organs (Escamilla-Trevino et al, 2006). The only investigated maize ortholog was not differentially expressed between RILs and F271. However, an Arabidopsis ortholog annotated *O-glycosyl hydrolase* (GRMZM2G119941) was dramatically less expressed in all RILs than in F271. In addition, the two investigated maize ABC transporters, encoding proteins putatively involved in the transport of monolignols across membranes (Miao et al, 2010; Kaneda et al, 2011), were not differentially expressed between RILs and F271.

Monolignol polymerization

While peroxidases have long been considered as the unique class of oxidases involved in lignin polymerization, dehydrogenative polymerization of monolignols is, in fact, driven by both peroxidases and laccases (Boudet, 2000; Onnerud et al, 2002; Boerjan et al, 2003; Tokunaga et al, 2009; Fagerstedt et al, 2010; Berthet et al, 2011). This has also been shown in maize (de Obeso et al, 2003; Caparros-Ruiz et al, 2006; Andersen et al, 2009). Peroxidases and laccases belong to large multigene families and it has been difficult to assign a specific function to any particular oxidase, especially because functional redundancy was often suspected. In Arabidopsis, only laccase double mutants clearly exhibited a modified lignin phenotype (Berthet et al, 2011). Nevertheless, the importance of oxidase redundancy could be partly reduced by the fact that many peroxidases or laccases have tissue specific expression patterns. When considering genes expressed in maize vascular and lignifying tissues (de Obeso et al, 2003; Caparros-Ruiz et al, 2006; Guillaumie et al, 2007b; Riboulet et al, 2009), at least five peroxidases and five laccases could be involved in monolignol polymerization. However, these latter numbers of members are likely under-estimated (Barrière et al, 2009). Among the four peroxidases for which expression data were available (Supplementary table 1B), the *ZmPox3* gene (GRMZM2G135108) was more expressed in all four RILs than in F271 at stage S1. In a partly contradictory way, a MITE insertion disrupting this gene was associated with higher cell wall degradability in a set of related European flint lines (Guillet-Claude et al, 2004). A similar tendency

towards a higher expression was also observed, except in RIL39, for *ZmPox2* (GRMZM2G040638), which was considered as involved in maize vascular vessels and epidermis lignification, at a greater extent than ZmPox3 (de Obeso et al, 2003). None of the four maize laccases described by Caparros-Ruiz et al (2006) were present on the Affymetrix array. However, two of three genes encoding for other laccases were more expressed in RILs than in F271 at stage S1. These latter correspond to two closely related genes (GRMZM2G132169 and GRMZM2G336337), with 93% sequence identity, which are orthologous to *AtLac12* (At5g05390). *AtLac12* is a gene expressed in Arabidopsis stems, mostly in the medium part, at a lower extent than *AtLac4* (At2g38080) and *AtLac17* (At5g60020) and (Berthet et al, 2011). Higher transcript levels of some peroxidases and laccases were found in lines exhibiting lower lignin content, mostly at emerging tassel S1 stage, possibly indicating a variable way of lignin polymerization between the selected RILs and F271. In addition, other peroxidases or laccases not present on the array could also have a determinant effect on lignin polymerization and structure.

Comparative expression of genes involved in regulation of lignin biosynthesis

MYB transcription factors

The regulation of phenylpropanoid and lignin biosynthesis was the first role identified for a plant R2R3-MYB transcription factor (Paz-Ares et al, 1987; Tamagnone et al, 1998). Later, comprehensive investigations have used the Arabidopsis model system, but also woody plants, documenting the hierarchical network including MYB regulating the secondary wall biosynthetic program (Goicoechea et al, 2005; Legay et al, 2007; Zhong et al, 2008; Wilkins et al, 2009; Zhong and Ye, 2009; Legay et al, 2010). In addition, MYB genes can be either transcriptional activators of lignin gene expression as was shown for *EgMYB2* (Goicoechea et al, 2005), *AtMYB46*, *AtMYB58*, *AtMYB85*, and *AtMYB103*, or transcriptional repressors as was shown for *EgMYB1* (Legay et al, 2007; Legay et al, 2010), *AtMYB4,* and *AtMYB86*. In maize, the two *ZmMYB40* and *ZmMYB95* duplicated genes (or *ZmMYB-IF35* and *ZMYB-IF25*) were shown to be involved in phenylpropanoid metabolism (Dias and Grotewold, 2003; Heine et al, 2007), but their involvement in regulation of monolignol biosynthesis is not yet established. Conversely, *ZmMYB31* and *ZmMYB42*, orthologous to barley *MYB-hv5* genes associated with vascular bundles (Wissenbach et al, 1993), were proven to be involved in the regulation of lignin pathway genes, with a repressive effect (Fornalé et al, 2006; Sonbol et al, 2009; Fornalé et al, 2010; Gray et al, 2012). Finally, the *ZmSWMYB46* (GRMZM2G052606) gene orthologous to *AtMYB46* was recently identified (Zhong et al, 2011). Several other MYB factors are surely involved in the regulation of maize lignification (Barrière et al, 2009, and unpublished data). Expression data were available for only a few of the *ZmMYB* genes putatively involved in maize cell wall lignification (Supplementary table 1C). Corroborating the probable involvement of other *ZmMYB* genes in secondary wall assembly in maize, the *ZmMYB* (GRMZM2G037650) located in bin 1.07, which is a EgMYB2-type and thus a putative positive regulator of transcription, was less expressed in RILs than in F271 at stage S1. Complementarily, the *ZmMYB* (GRMZM2G017520) located in bin 3.04, which is a *EgMYB1-type* and thus a putative negative regulator of transcription, was more expressed in all four RILs at the two stages. However, and conversely, the *ZmMYB* (GRMZM2G041415) located in bin 3.05, ortholog of negative regulators of gene transcription, was less expressed in three RIL at stage S1, and in all RILs at stage S2.

NAC transcription factors

Lignin pathway genes are regulated upstream of the MYB transcription factor level by NAC transcription factors. A set of NAC domain transcription factors (SND1, NST1, VND6, VND7) has, thus, been shown to act as a positive master regulator of secondary cell wall biosynthesis in Arabidopsis (Yamaguchi et al, 2008; Zhong et al, 2008; Zhong and Ye, 2009; Zhou et al, 2009; Zhong et al, 2010). Orthologs of these NAC are present in other plant species, but no exhaustive information is available in maize. Several maize orthologs are, nevertheless, available from Barrière et al (2009), Shen et al (2009), and Zhong et al (2011) data, even if some of these NAC are possibly involved in other pathways than cell wall assembly. Based on data available for 13 *NAC* genes putatively involved in regulation of cell wall lignification, no differences in expression were clearly shown between RILs and F271 (Supplementary table 1C). There was, nevertheless, a tendency to a higher expression in the four RILs for one *NAC* (GRMZM2G176677) located in bin 2.03 at stage S2, and also in three RILs at the two stages for one *NAC* (GRMZM2G456568) located in bin 6.05. A lower expression was also shown for one NAC located in bin 7.04 in three RILs at stage S2.

KNAT7 orthologous genes

KNAT7 was shown to be a transcriptional repressor of secondary wall biosynthesis, involved in "a negative feedback loop that maintains cellular homeostasis during developmental commitment to secondary cell wall deposition" (Li et al, 2012). However, none of the *KNAT7* orthologs for which data were available were differentially expressed between RILs and F271 (Supplementary table 1C).

Comparative expression of genes involved in regulation of lignified tissue assembly

Genes involved in the regulation of lignified tissue assembly are located upstream in pathways potentially regulating the plant final lignin content or cell wall degradability. It is, thus, questionable whether or not these genes could be good candidate genes explaining the effect of the detected QTLs. In fact, their

involvement could be considered if observed differences in lignin content or cell wall degradability are related to variation in areas and patterning of lignified tissues. Such large differences were shown between lines, with lines of which parenchyma is not lignified, or with large differences in lignified tissues surrounding vascular bundles, or large variation in width of the lignified cortex (Méchin et al, 2005; Riboulet et al, 2008b).

Zing finger encoding genes

Zinc finger proteins, which constitute one of the largest families of transcription factor regulatory proteins, are involved in the regulation of numerous elementary processes of plant development, including lignified tissue assembly. Among zinc-finger, members of the small class III homeodomain leucine-zipper (HD-ZIP) proteins have been shown to play a regulatory role in vascular differentiation, as illustrated by the investigations on the *IFL1* (I*nterfascicular Fiber Less1*, synonymous *Revoluta*) genes of Arabidopsis or *Rolled Leaf* (*RLD1* and RLD2) genes in maize (Zhong and Ye, 1999; Ratcliffe et al, 2000; Juarez et al, 2004). The *RLD1* gene is regulated by the miR166 (Juarez et al, 2004), and the expression of the aspen *PtaHB1* gene, orthologous to *IFL1* and *RLD1*, which was closely associated with wood formation, was inversely correlated with the level of miR166 miRNA (Ko et al, 2006). In addition, interactions between *HDZIPIII* 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 HDZIPIII and KANADI transcription factors control cambium activity, with KANADI proteins acting on auxin transport, and HDZIPIII proteins promoting axial cell elongation and xylem differentiation (Ilegems et al, 2010). Because there is no secondary cambium in grasses, the latter *HDZIPIII* and *KANADI* genes, obviously, have different functions than in Arabidopsis or aspen. Notwithstanding, the rolled-leaf mutations have consequences in lignified tissue patterning, even if no difference in expression of these genes was observed between F271 and any of the four RILs (Supplementary table 1D).

While the CCCH zinc finger protein (AtC3H14) may function as master regulator of secondary wall biosynthesis and activate all of the secondary wall phenolics and carbohydrate related genes tested (Ko et al, 2009), no clear involvement of C3HC4 RING finger proteins in regulation of lignin-related metabolism has been reported so far. RING finger proteins are involved in numerous cellular processes including transcription regulation, signal transduction, protein-protein interaction and ubiquitination (Ma et al, 2009). However, the *C3HC4 zinc finger* (GRMZM2G062724), located in bin 1.01, was more highly expressed in the four RILs than in F271, especially at the earlier S1 stage. In addition, the C3HC4 zing finger GRMZM2G077307, located in bin 3.07, was underexpressed in three RIL.

Members of the LIM protein family contain zinc-binding domains. Unlike the classical zinc fingers, these domains do not bind DNA, but mediate interactions with other proteins (Matthews et al, 2009). The tobacco *NtLIM1* gene was shown to be a positive regulator of the lignin pathway (Kawaoka and Ebinuma, 2001). The closest maize ortholog of *NtLIM1* (GRMZM2G004959) has no probe on the Affymetrix array. Expression data were only available for two paralogs. The most expressed LIM gene is annotated in the maizesequence database as encoding the "pollen specific protein SF3" which was, in fact, clearly expressed in stalks. No differences in LIM gene expression were shown between RILs and F271.

bZIP GRAS SCARECROW-like genes

The GRAS SCARECROW and SCARECROW-like proteins belong to a plant specific transcription factor family which contains basic leucine zipper regions (Di Laurenzio et al, 1996; Lee et al, 2008). These proteins are involved in complex regulatory pathways regulating tissue patterning and differentiation. The SCARECROW protein is involved in bidirectional cell signaling mediated by miRNA165/6 and interfering with the transcription factor SHORT ROOT (SHR, equally expressed in stem and root) and class III homeodomain leucine zipper proteins towards the control of xylem patterning (Carlsbecker et al, 2010). Out of the four maize orthologs with available expression, the one located in bin 2.07 (GRMZM2G431309) was more expressed in three RILs, while the one located in bin 6.05 was more expressed in two RIL, at stage S2 for the two genes.

COV-like genes

The *COV1* (*CONTINUOUS VASCULAR RING*) recessive mutant of Arabidopsis has a great increase in stem vascular tissue at the inter-fascicular regions (Parker et al, 2003). In addition to the *COV1* gene (At2g20120), two *COV1-like* genes (*LCV2*, *Like-COV-2*, At1g43130, and *LCV3*, *Like-COV-3*, At2g18460) are present in the Arabidopsis genome. Ten genes encoding potentially for COV orthologs were found in the maize genome, but probes and/or data were available for only four genes, without differences in expression between RILs and F271.

EgROP1-like genes

A member of the plant ROP family (*EgROP1*) was shown to be preferentially expressed in the cambial zone and differentiating xylem of eucalyptus. Its overexpression in Arabidopsis altered vessel formation and fiber growth in secondary xylem (Rengel et al, 2009; Foucart et al, 2009). None of the three maize orthologs of *EgROP1* with available probe data was differentially expressed between RILs and F271.

Microtubule-Associated Proteins

Two microtubule-associated proteins (AtMAP70-5 and AtMAP70) were shown in Arabidopsis to be essential for defining where secondary cell wall polymers are applied at the cell cortex in wood-forming cells (Pesquet et al, 2010). There was higher expres-

sion of one maize ortholog (GRMZM2G832989), located in bin 5.06, in three RILs compared with F271, especially at the later S2 stage.

Differentially expressed genes underlying the major QTL position (bin 6.06)

As was previously quoted, only two (or three) genes, with a possible role in secondary wall biosynthesis, were differentially expressed between RILs and F271. The NAC transcription factor located in bin 6.05 could be an upstream regulator of cell wall assembly, but its higher expression concerned only three RILs out of four, a fact that did not convincingly designate it as a good candidate gene for the studied QTLs. A higher expression was also shown, but only at stage S2, for the *OMT ZRP4-like5c/5d* gene(s). Even if the *OMT-ZRP4* genes have been considered with a possible involvement in methylation of aromatic ring during cell wall phenolic compounds biosynthesis (Barrière et al, 2009), a differential expression only at stage S2 is not the most probable situation for a candidate gene with major effect. In addition, nine genes located in bin 6.06 QTL support intervals were differentially expressed between RILs and F271, including four genes of unknown function (Table 4).

Alpha-expansin gene

The α-*expansin 5* (*EXPA5*), has a functional annotation which is also related to cell wall metabolism. Expansins appear to be 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, 2003). While the rice genome contains at least 28 α-expansin genes (Shin et al, 2005), the whole number of α-expansin genes is not yet known in maize. Ten genes are annotated alpha-expansin in the maize sequence database, nine of them belonging to a group of 38 paralogs, allowing a probable redundancy in α-expansin activities. However, the *EXPA5* gene (GRMZM2G361064) located in bin 6.06 (position 154.73 Mbp) appears different from the others as it is the only one without any paralogs. This *ZmEXPA5* gene was expressed in region of leaves where elongation has ceased and where secondary cell wall deposition occurred (Muller et al, 2007). In addition, 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) and expansins similarly appeared to be expressed during the differentiation of the tracheary elements, suggesting their possible involvement in secondary wall formation or/and primary wall disassembly (Milioni et al, 2001). The *EXPA5* gene was, at least, expressed 20 times more in RILs than in F271 at stage S1 and S2, except in two RILs at stage S2 with an only three times greater expression of this gene.

FKBP gene

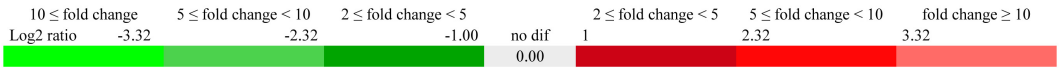
FK506-binding proteins (FKBPs) belong to the large family of peptidyl–prolyl *cis-trans* isomerases, which are known to be involved in many cellular processes, such as cell signaling, protein trafficking and transcription (Harrar et al, 2001). A putative ortholog, GRMZM2G035922, in maize genome located in position 151.5 Mbp was less expressed in RILs than in F271, especially at stage S2. One gene of this family, *Pasticcino1* (*PAS1*), was shown to play an important role in the control of plant development, controlling cell division or differentiation. The PAS mutants of *Arabidopsis thaliana* show ectopic cell proliferation in cotyledons, extra layers of cells in the hypocotyl, and an abnormal apical meristem (Vittorioso et al, 1998).

Seed maturation protein reticulon (RTN) family

The reticulon family gathers a large group of membrane-associated proteins found throughout the eukaryotic kingdom. Reticulons principally localize to the endoplasmic reticulum, and they are involved in

Table 4 - Differentially expressed genes between F271 and four RILs underlying the major QTLs located in bin 6.06, in below-ear internodes, at two growing stages, S1 = emerging tassel stage and S2 = silking stage. GRMZM numbers are given according to the release 5b.60 of the www.maizesequence.org database. Range of colors according to the level of differential expression between F271 and RILs, a log2 sum intensity in green or in red indicates that the gene is less expressed or more expressed in RILs, respectively, other log2 sum intensities (not in green or red) were not found to be statistically significant after Bonferroni correction ($P < 0.05$).

Gene	GRMZM gene	bin	Pos.	Emerging tassel stage (S1)					Silking stage (S2)				
			Mpb	F271	RIL39	RIL99	RIL54	RIL118	F271	RIL39	RIL99	RIL54	RIL118
FK506 binding protein	GRMZM2G035922	6.05	151.49	9.95	9.02	8.91	8.44	8.92	10.52	8.94	9.13	8.86	9.03
Unknown function	GRMZM2G125037	6.05	153.76	3.13	6.87	6.16	5.49	5.35	3.08	3.96	3.77	4.24	3.70
Alpha-expansin 5	GRMZM2G361064	6.06	154.13	3.49	7.07	8.58	8.97	7.92	2.61	7.18	4.40	7.21	4.84
Unknown function	GRMZM2G037111	6.06	154.99	6.36	3.41	3.12	3.22	3.12	6.59	4.70	3.22	4.59	3.20
Unknown function	GRMZM2G129166	6.06	155.07	4.52	8.71	9.41	9.11	10.00	3.73	8.44	8.98	8.85	9.16
Unknown function	GRMZM2G315199	6.06	156.12	7.33	7.26	7.50	8.34	8.33	5.86	8.13	7.44	7.66	7.77
Heat shock 60kDa protein	GRMZM2G074790	6.07	161.73	2.86	2.37	2.46	2.36	2.28	4.07	2.39	2.35	2.56	2.34
Seed maturation protein	GRMZM2G004828	6.07	162.64	7.49	2.25	4.03	4.38	3.21	7.04	2.17	2.17	2.16	2.16
Non-specific lipid-transfer protein	GRMZM2G320373	6.07	162.66	9.18	9.33	9.68	9.24	9.36	8.96	7.29	5.81	5.36	7.27



numerous cellular processes including apoptosis, cell division, and intracellular trafficking, vesicle formation and membrane morphogenesis (Yang and Strittmatter, 2007; Nziengui and Schoefs, 2009). One member, GRMZM2G004828, annotated seed maturation RTN and located at the distal part of QTL support interval, was less expressed in RILs than in F271 at both stages. This result also pointed out an expression and a possible role of members of this gene family in growing stems.

LTP encoding genes

Plant lipid transfer proteins (LTP) are small, abundant lipid-binding proteins of biological, mostly unknown, functions (Arondel et al, 2000). Various biological roles for plant LTP have, nevertheless, been proposed, including defence against pathogens and modulation of plant development (Boutrot et al, 2008). In addition, xylogen is an extracellular arabinogalactan protein (AGP) which has a unique structure, containing a non-specific lipid transfer protein (nsLTP) domain and AGP domains which are involved in vascular development (Sieburth and Deyholos, 2005; Kobayashi et al, 2011). One member of the LTP family (GRMZM2G320373, position 162.66 Mbp) was less expressed in RILs than in F271 at stage S2.

Heat shock 60kDa protein

Heat shock proteins (Hsps) were first identified as proteins whose synthesis was enhanced by stresses. Recently, several major Hsps have been referred to as molecular chaperones of protein biogenesis. Hsp60 binds to unfolded proteins, preventing aggregation and facilitating protein folding (Craig et al, 1993). The GRMZM2G074790 gene located in position 161.73 Mbp was less expressed in RIL than F271 at stage S2, but its involvement in a pathway inducing differences in lignin traits did not appear very likely.

Genes of unknown function

The most differentially expressed gene (GRMZM2G129166, position 155.07 Mbp) in bin 6.06 was located a little downstream of the position of *EXPA5* gene. This gene, of unknown function, was more highly expressed in RILs than in F271 at both stages S1 and S2. Another gene of unknown function (GRMZM2G361064) located in position 154.99 Mbp was less expressed in RILs at the two stages. Finally, two other genes of unknown function were more expressed in RILs than in F271, one at stage S1 (GRMZM2G125037, position 153.76 Mbp), and one at stage S2 (GRMZM2G315199, position 156.12 Mbp).

Differentially expressed genes without known cell wall phenolic compound relationships and underlying QTL positions in bins 1.02 and 3.05

Thirty six genes located in the support intervals of the two other cell wall related QTL detected in the F288 x F271 progeny (bins 1.02 and 3.05) were differentially expressed between RILs and F271 (Supplementary tables 1E and 1F). Five and three of them, located in bin 1.02 and 3.05, respectively, had no described function. While several of the differentially expressed genes were related to cell metabolism, signal transduction, and protein catabolism [serine/threonine kinase, RNA recognition motif (RRM) containing protein, F-box domain containing protein, guanine nucleotide-binding (G) proteins, hydroxymethylglutaryl-CoA synthase], several others had functions which could putatively be related to the phenotypic differences between F271 and the four RILs.

As other glutathione S-transferases, the *GST6* (GRMZM2G096247) and *GST3* (GRMZM2G146246) genes, located in bin 1.01 and 3.05, respectively, likely has a coupled activity with ABC transporters (Yazaki, 2005) and could be involved in transport of phenolics compounds used, or not, in cell wall assembly. These two GST were less expressed in RILs at stage S2. Exostosin-like proteins, which were initially described as encoded by a family of human tumour suppressor genes, are trans-membrane glycosyltransferases found in the endoplasmic reticulum. Such glycosyltransferases might also be involved in plant cell wall carbohydrate metabolism, such as the GT47 family xylan xylosyltransferase members that were hypothesized to play a role in the elongation of the xylan backbone (Brown et al, 2009; Wu et al, 2009). One *exostosin* gene (GRMZM2G158496) located in bin 3.05 was more expressed in RILs. Dihydroflavonol 4-reductases (DFR) are involved in the phenolic compound pathway and catalyze the reduction of dihydroflavonols to leucoanthocyanins. Leucoanthocyanidins can be utilized for the synthesis of compounds, such as catechins and oligomeric proanthocyanidins, which have anti-oxidant effects. The *DFR* gene (GRMZM2G109589) located in bin 1.02 was less expressed in RILs.

Pathogenesis-related proteins (PR proteins) were first defined as proteins that are not detectable in healthy tissues (or only at basal concentrations), but for which protein accumulation was shown upon pathological and stressed conditions. Although some PR proteins exhibit potential in vitro antimicrobial activities, a direct functional role in defense could not be demonstrated for all (Sels et al, 2008). Their expression in healthy tissue likely indicated other involvement in plant metabolism, and the two *PR* genes (GRMZM2G112538 and GRMZM2G112524) located in bin 1.03 were less expressed in RILs, especially at stage S2. Among PR proteins, γ-thionins were the first plant defense proteins described in literature, with growth inhibition activity toward pathogens (Pelegrini and Franco, 2005). A flower-specific γ-thionin gene (GRMZM2G392863), located in bin 1.01, was less expressed in RILs and F271 at stage S2, indicating that such proteins are expressed elsewhere than in flowers, with roles differing from plant defense (a false annotation could also be considered). In addition, while chitin has been considered as an inducer for defence mechanisms in plants, the lower expression of an endochitinase gene (GRMZM2G099454) in RILs could

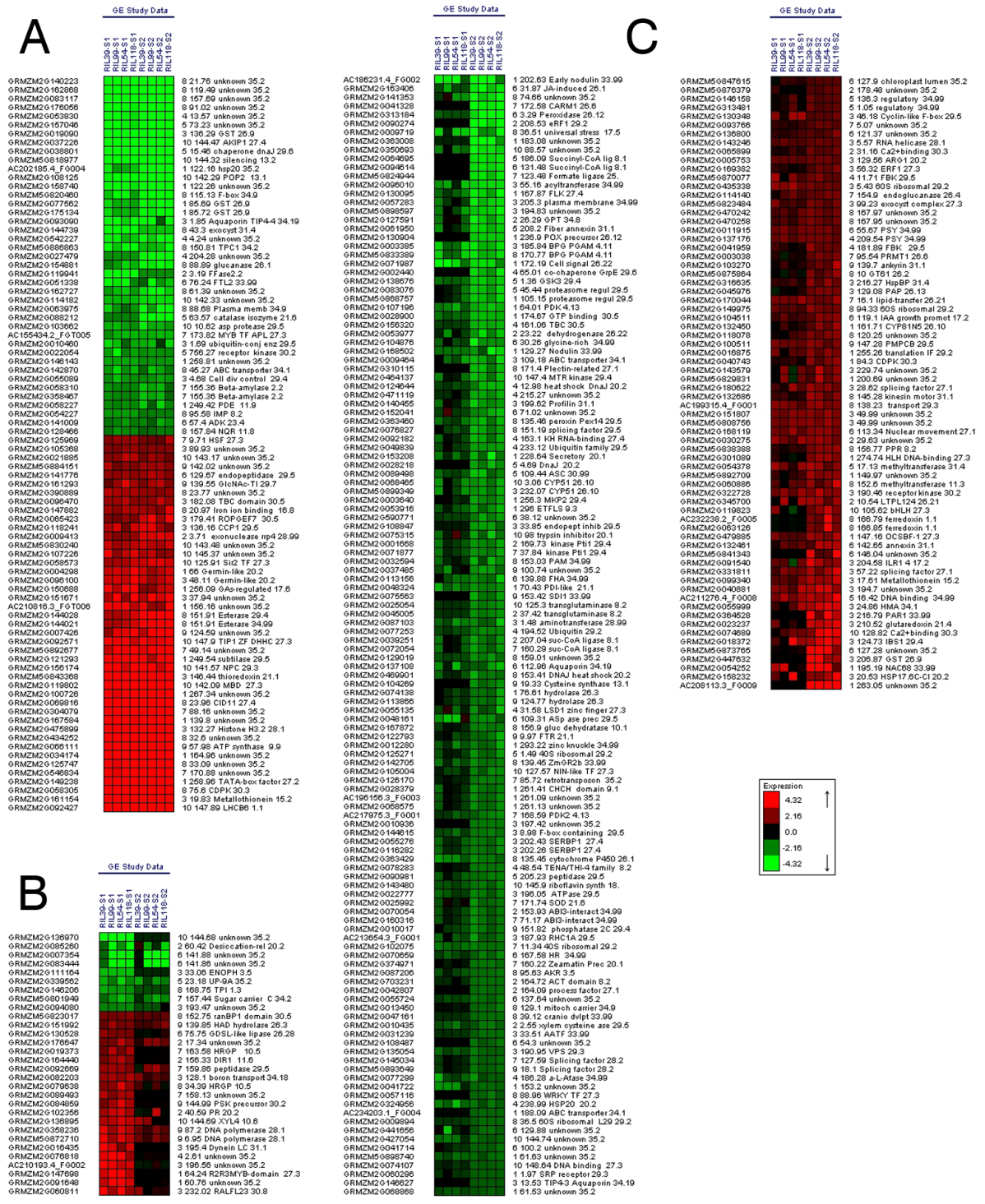


Figure 3 - Differentially expressed genes between F271 and the four RIL without known cell wall phenolic compound relationships and located elsewhere than in considered QTL positions. GRMZM numbers are given according to the reference genome B73 (Schnable et al, 2009, www.maizesequence.org release v2). Text on the right correspond to chromosome number, physical position in Mbp, annotation and Mapman bincode. (A) Differentially expressed at the two S1 and S2 stages. (B) Differentially expressed only at "emerging tassel" stage (S1). (C) Differentially expressed only at "silking date" stage (S2).

likely indicate a different function.

The CLAVATA1 (CLV1) receptor kinase regulates stem cell specification at shoot and flower meristems of Arabidopsis (Diévart et al, 2003; Durbak and Tax, 2011). CLV proteins restrict the expression domain of the stem cell-promoting homeodomain transcription factor WUSCHEL (van der Graaff et al, 2009). Similarly, *FWL* genes are a large gene family in plants, but with a strong conservation of key domains, suggesting a conservation of the core biochemical function of these proteins (Guo et al, 2010; Libault and Stacey, 2010). In maize, putative *fw2.2* orthologs were identified as *Cell Number Regulator* (*CNR*) genes, including the two closest orthologs *CNR1* and *CNR2*. Overexpression of *CNR1* reduced overall plant size while plant and organ size increased when its expression was co-suppressed or silenced, with changes in cell number, but not in cell size. *CNR2* expression was found to be negatively correlated with tissue growth activity and hybrid seedling vigor (Guo et al, 2010). The *CLV1* gene (GRMZM2G043584), located in bin 1.03, was more expressed in the four RILs at the two stages. Such differential expressions of genes putatively related to tissue differentiation and patterning could induce differences in lignified tissue areas and, consequently, on cell wall degradability.

Differentially expressed genes without known cell wall phenolic compound relationships and located elsewhere than in bins 6.06, 1.02 and 3.05 QTL positions

Finally, 312 differentially expressed genes between F271 and the four RIL were located elsewhere than in the three considered QTL positions and were not yet known to be involved in cell wall biosynthesis (Figure 3).

Highly differentially expressed genes

Forty two of these genes were more than 20 times differentially expressed between the four RILs and F271. No annotation was available for 18 of them. Among the 24 annotated genes, some genes could be more specifically considered as putatively involved in the differences observed between F271 and the four RILs, or with questionable role in maize. The *glutathione S-transferase GST18* (GRMZM2G019090) was less expressed in RILs at the two stages, and could be involved in the transport of phenolics compounds. The *pollen pistil incompatibility POP2* gene (GRMZM2G108125), less expressed in RILs at the two stages, encodes a transaminase that degrades GABA (Palanivelu et al, 2003), of which accumulation causes cell elongation defects and a decrease in expression of genes encoding cell wall-related proteins in *Arabidopsis thaliana* (Renault et al, 2011). The *Rapid Alkalinization Factor RALF23* (GRMZM2G060811), more expressed in RILs at stage S1, was shown to be down-regulated in the presence of brassinolide and over-expression of *AtRALF23* reduced plant growth, counteracting brassinolide growth-promoting effects (Srivastava et al, 2009). AtRALF23 is synthesized as a larger precursor protein, and is released in peptide form by the action of AtS1P, a subtilisin serine protease. One *subtilase* (GRMZM2G121293) was also more expressed in RILs at the two stages. The coactivator-associated *arginine methyltransferase, CARM1* (GRMZM2G041328), under-expressed in RILs at stage S2, modulates maintenance of embryonic stem cell pluripotency and early embryonic development in animal systems (Zhao et al, 2011). The homologous *AtPRMT4a* and *AtPRMT4b* genes were shown to be involved in regulation of flowering time (Niu et al, 2008) while the *AtPRMT5* is involved in vegetative growth and flowering time (Pei et al, 2007). The *eukaryotic peptide chain release factor subunit1-1 eRF1* (GRMZM2G090274), less expressed in RILs at stage S2, was shown to be involved in cell elongation and radial cell division. Its co-suppression in Arabidopsis has a major effect on plant morphology, with a reduction in internode elongation, reduced height of cells, and ectopic lignification (Petsch et al, 2005). Moreover, cell division in the fascicular cambial regions was altered. In addition, four other differentially expressed genes had probable involvements in transcription (*TATA box factor*, GRMZM2G149238), regulation of transcription (MBD *Methyl-CpG binding domain containing protein*, GRMZM2G119802) or RNA binding proteins (*AKIP1*, GRMZM2G037226, and *CTC-Interacting Domain CID11*, GRMZM2G069816).

Other differentially expressed genes

Among the genes less than 20 times differentially expressed between F271 and the four RILs, four genes were classified in the cell wall class, two genes encoding hydroxyproline-rich glycoprotein (HRGP), one gene encoding hydrolase XYL4 hydrolyzing O-glycosyl compound, and one gene encoding a dTDP-glucose 4,6-dehydratase, probably involved in cell wall metabolism. Two other *GST* and three other ABC transporters were also less expressed in RILs. The *TIP GROWTH DEFECTIVE1 S-acyl transferase* (*TIP1*) was strongly differentially expressed at the two stages in RILs. This gene regulates plant cell growth in Arabidopsis (Hemsley et al, 2005). Moreover, a *MYB* (AC155434.2_FGT005) orthologous to arabidopsis *MYR1* (*MYB related protein1*) and one *NAC* (GRMZM2G054252), putatively involved in cell division, were differentially expressed between the four RILs and F271. Finally, six genes, putatively involved in cell organization, were also differentially expressed, including the *annexin* (GRMZM2G061950) which was ortholog of Atg38760, a target of SND1 (Zhong et al, 2010).

Discussion

A lot of genes were shown differentially expressed between F271 and the four RILs, even if the main limit of such a transcriptomic approach using a 18,000 probes Affymetrix micro-array is obviously related to the fact that only nearly one third of the maize genes are present on the array. Several genes involved in

monolignol biosynthesis and polymerization, and a lot of transcription factors putatively in secondary wall biosynthesis and lignified tissue assembly, could thus not be investigated. In addition, differences between cell wall traits in lines could be related to genetic variation giving no difference in expression, such as variation in protein sequence and efficiency. Moreover, differences could be related to variation in regulation by miRNA, even if no miRNA related to lignification has yet been identified in bin 6.06.

Among the differentially expressed genes located in bin 6.06, the closest genes to QTL peak position were three genes, which encode proteins of unknown function (GRMZM2G037111, GRMZM2G129166 and GRMZM2G315199). None of the other genes could be easily considered as candidates underlying the effect of this detected QTL, except the *FKBP* gene and the α-*expansin 5* gene even if these genes were not clearly related to lignification but to cell division and extension, respectively. The differential expression could also be related to allelic variation in these genes and/or their co-regulation.

Only a few genes located outside the QTL positions in bin 6.06 and with a function related to cell wall assembly were differentially expressed between the four considered RILs and F271. Among genes involved in monolignol biosynthesis, a differential expression was only shown for the *ZmPAL2b*. Complementarily, two *ZRP4-like OMT*, one *ZmPox*, two *ZmLac*, and one *glycosyl hydrolase* gene were also differentially expressed in RILs and in F271. Their variable expression, related or not to a regulation factor located in bin 6.06, could contribute to explain the observed differences.

Three *ZmMYB* were differentially expressed when comparing the four RILs to F271. The *ZmMYB* located in bin 1.07, which is orthologous to AtMYB85 and EgMYB2 and positively regulates gene transcription, colocalized with several major lignin-related QTLs shown in the F838 x F286 progeny (Barrière et al, 2008; Barrière et al, 2010). Similarly, the *ZmMYB2* and the *ZmMYB8* located in bin 3.04 and 3.05, which is orthologous to AtMYB86 and AtMYB4 respectively and negatively regulates gene transcription, colocalized with several lignin-related QTLs shown in the RIo x WM13 progenies (Barrière et al, 2012). They are also located upstream of other QTLs shown in bin 3.05 in the F288 x F271 progeny (Roussel et al, 2002; Thomas et al, 2010). These three *ZmMYB* could not be considered as the primary determinants of the observed variation as they are not colocalized with the major QTLs of the F288 x F271 progeny. However, the observed differences in expression strengthened their plausible involvement as target of an upstream regulation factor potentially colocalizing with the major QTL under investigation. Similarly, the differentially expressed *C3HC4 zinc finger* could be considered as a target factor. This gene was underlying QTLs shown in the F288 x F271 progeny (Roussel et al, 2002; Thomas et al, 2010) and was also in close position, even a little upstream, to QTLs shown in the F838 x F286 progeny (Barrière et al, 2008). In addition, the differentially expressed NAC was localized under QTLs of two RIL progeny (F7025 x F4 and FI16 x F2).

Finally, among the other genes differentially expressed, some genes were involved in plant growth in particular in the cell division and elongation. Such genes could be considered as probable candidates even if the modifications engendered by these genes have not yet been proved to be involved in cell wall lignification and degradability.

Conclusions

No definite conclusion was available from the current expression study for the discovery of the candidate gene(s) underlying cell wall related QTLs located in bin 6.06. In addition, and relative to the probes spotted on the array, the determinant did not appear as one of the genes previously listed as putatively involved in cell wall assembly. Corroborating results obtained on Arabidopsis, genes involved in monolignols biosynthesis are very likely not candidates underlying major QTLs for lignin content or cell wall degradability, despite the major effect of mutations in *ZmCCR1*, *ZmCOMT*, or *ZmCAD2* genes. Variation in expression of monolignol-related genes could, nevertheless, be a consequence of upstream regulation factors inducing differences in lignin content, structure and composition.

Observed differential gene expression between the four RILs and F271 was certainly partly not related to cell wall related traits, and was the consequence of allelic variations and linkage disequilibrium, even if several of them were likely co-regulated with, or targeted by, the primary trait determinants. In addition, a few genes could be considered as new members involved in secondary wall assembly. The differential expression of the three *ZmMYB* and the *C3HC4 zinc finger* could be considered with a reasonable level of confidence as related to variation in cell wall traits between F271 and the four RILs and, possibly, as a consequence of the genomic variation in bin 6.06 area. Assuming this hypothesis was true, the QTL underlying genetic determinant would, thus, be a gene involved in an upstream mechanism of lignin biosynthesis and lignified tissue assembly. In addition, the involvement of genes of still unknown function has likely to be considered.

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