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Investigating the unusually high cell wall digestibility of the old INRA early flint F4 maize inbred line

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Abstract

The old INRA flint early line F4, which belongs to the northern flint group, is typified by its high cell wall digestibility which reaches values as high as those observed in several brown-midrib bm3 mutant lines. The F4 line thus appeared as a model that could contribute to the understanding of genetic mechanisms involved in variation of secondary wall traits. Different strategies and results were thus gathered including especially cell wall biochemical and digestibility investigations, expression approaches, QTL investigations, and colocalizations between QTLs and candidate genes. Lignin content was lower in F4 than in other lines, with a tendency to lower *p*-coumarate content. The Syringyl/Guaiacyl lignin unit ratio was similar in F4 as in other lines, but this ratio was nearly not reduced in F4bm3, conversely to what is observed in *bm3* mutants. In comparison with the INRA F2 control line, expressions of three PAL genes including the *ZmPAL*, of the *ZmF5H1* and the *ZmCOMT* genes were significantly reduced in F4 lignifying ear internodes at early silking stage. In the F7025 x F4 RIL progeny, seven QTLs were shown with favorable alleles (increasing cell wall digestibility) originating from F4. Two strong QTLs were located in bins 1.03 and 2.03 colocalizing with the *ZmMYB019* and *ZmSWN6* transcription factors, respectively. Orthologs of *ZmMYB019* have been shown to be involved in lignin biosynthesis, and the *PpMYB8* ortholog was shown to regulate PAL gene expression in maritime pine. The *ZmSWN6* NAC transcription factor is an upstream master regulator of the secondary wall biosynthetic programs. At the other QTL positions, colocalizations were also shown with other secondary wall related ZmMYB, but also with BAHD genes involved in arabinoxylan feruloylation, and with the position of the bm6 mutation. Three QTL positions were shown with favorable alleles originating from F7025, which colocalized with ZmMYB and ZmNAC transcriptions factors. As a tentative conclusion, the F4 unusually high cell wall digestibility is likely greatly related to the altered working of at least two major transcription factors regulating cell wall biosynthesis and assembly.

Keywords: *maize, cell wall, lignin, digestibility, QTL, gene expression, MYB, NAC, brown-midrib*

Introduction

Maize was early recognized as an excellent forage plant, soon after its introduction in Europe. It was, for long time, considered as other common forage grasses and mostly used as fresh green fodder for animal feeding during summer and early autumn. Forage conservation by ensiling was explored in second part of the 19th century, but the major use of silage maize for cattle feeding began only one hundred years later with the development of early flint x dent hybrids tolerant to low spring and early autumn temperatures. Forage maize is now the most important annual forage crop in northern Europe. Despite the fact that maize silages provide roughages with average high energy contents, large genetic variations in energy value were shown between maize genotypes (early and medium early) ranging from 0.78 to 0.97 UFL (UFL is French energy forage unit for milk production, with 1 UFL = 7.11 MJ). Variations in energy values were shown to be primarily related to variations in cell wall digestibility. When measured *in vivo* with sheep in digestibility crates, cell wall digestibility, which is thus the underlying main determinant of silage maize energy value, ranged from 36 to 60% (Barrière et al, 2004a; INRA Lusignan, unpublished data).

In Europe, as well as in North America, most resources that were first used in forage maize breeding comprise germplasm bred and also used in grain maize hybrids, all the more as the silage specificity was not soon considered. During the 1960 to 2015 period, there was a tremendous improvement of whole plant yield of released hybrids, nearing an average 0.20 t ha^{-1} $year^{-1}$, with simultaneous great

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improvement in stalk standability and breakage resistance. However, especially in the 1990 decade, a significant part of released hybrids were of lower cell wall digestibility than most of older ones. Numerous alleles contributing to cell wall digestibility and high feeding value were eliminated during breeding for higher agronomic values, or were lost because of genetic drift (Barrière et al, 2004a). The specific silage maize breeding programs and registration rules that have been established in France nearly 20 years ago, with the taking into account of traits related to feeding value in cows, have stopped the decrease in hybrid energy value. Silage maize hybrids registered since about 15 years have thus high yield, high standability, with feeding values that are similar to those of hybrids of late 1970 and early 1980 periods. These current modern hybrids have nevertheless cell wall digestibility and energy values that are still lower than the ones of the best older hybrids (-5 to -7%), but with major whole plant yield improvements (30 to 50%).

Breeding of early and medium early (silage) maize has been a long time based on the «flint x dent» heterotic scheme. From 1957, with the registration of INRA200, to 1979, flint lines were mainly related to the INRA F2 and F7 lines originating from the Lacaune landrace, to the Spanish line EP1, and, mostly in Germany and northern Europe, to progenies of the «Gelder Badisher Landmais (Bade Yellow)» landrace (Barrière et al, 2006). During this period, dent lines were mostly related to the Wisconsin and Minnesota germplasms. From 1980 and the registration of DEA and DEA-type single-way hybrids, to the early 1990s, hybrid flint lines were mostly often F2 or related to F2 while dent lines belong or were related to the Iodent germplasm. Afterwards, a broadening of the genetic base of early flint lines occurred through the introgression of eastern Europe and Canadian flint, (medium) late dent, and tropical germplasms (Barrière et al, 2005). The progressive disappearance of the F7 and related lines from hybrid pedigrees at the end of the 1970 years dated the beginning in the average decrease of cell wall digestibility in early hybrids (but simultaneously dated the increasing progresses in agronomic value). More recently, medium early and an increasing part of early hybrids result from mostly dent heterotic patterns, including Iodent, BSSS, Lancaster, and original proprietary germplasms.

In silage maize hybrids, a low cell wall digestibility is all the more detrimental to cattle that it also significantly impedes the silage intake. The search for more digestible and ingestible maize, allowing future progresses in feeding value, also requires investigating genetic resources that were not used in grain maize breeding, especially in order to discover the underlying determinants of feeding values traits. Because a large gap in agronomic value exists between these resources possibly of interest and elite modern lines, specific strategies of introgression of favorable alleles for feeding value traits into elite germplasm have to be used and are currently available, from marker assisted selection to gene editing with the Crispr/Cas9 technology. Among resources of interest in breeding for high silage maize feeding value, the old INRA flint line F4 appeared as a model because its unusually high cell wall digestibility, reaching value as high as those observed in several brown-midrib bm3 lines (Méchin et al, 2000; Fontaine et al, 2003; several unpublished INRA Lusignan data). Gathering data available on this line could thus contribute to understand the reasons why F4 secondary walls are so digestible and simultaneously to highlight significant determinants involved in maize cell wall digestibility variation. Breeding for silage maize hybrids with significantly improved cell wall digestibility and energy value is a major way towards reducing concentrate amounts in dairy cow diets, leading thus to less costly, more efficient and more sustainable cattle feeding strategies.

Materials and Methods

Feeding value and plant component estimates

In both line comparison experiments and QTL investigations in RILs *per se* value and topcrosses, feeding value traits were estimated as previously described (Barrière et al, 2008). Neutral Detergent Fiber (NDF) and Acid Detergent Lignin (ADL) were estimated according to Goering and van Soest (1970), Klason lignin (KL) according to Dence and Lin (1992), and the *in vitro* dry matter digestibility (IVDMD) according to Aufrère and Michalet-Doreau (1983). Cell wall digestibility was estimated according to Struik (1983) and Dolstra and Medema (1990) as the *in vitro* NDF digestibility (IVNDFD) computed assuming that the non-NDF part of plant components was fully digestible [IVNDFD = 100 x (IVDMD - (100 - NDF)) / NDF].

In line comparison experiments, *p*-hydroxycinnamic acid contents were measured after a mild alkaline treatment allowing the release of esterified ferulate (esterFA) and *p*-coumarate (pCA), and a severe alkaline treatment allowing the release of etherified ferulate (etherFA) according to the procedure previously described (Morrison et al, 1993; Méchin et al, 2000). During oxidation of cell wall residues with alkaline nitrobenzene, *p*-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) lignin monomers are oxidized into *p*-hydroxybenzaldehyde (pHb), vanillin (Va) and syringaldehyde (Sg), respectively (Roadhouse and MacDougall, 1956; Higuchi and Kawamura, 1966). During alkaline nitrobenzene oxidation, a part of *p*-coumarate and a part of ferulate are also oxidized into pHb and Va, respectively, and amounts of *p*-hydroxyphenyl and guaiacyl units of lignins can thus be a little over-estimated.

For all investigated traits, due to the large number of samples, all estimates were done using previously described near infrared reflectance spectroscopy (NIRS) calibrations (Barrière et al, 2008), with validations of corresponding calibration equations based

on laboratory analyses of about 30 samples per year and location.

QTL analysis in the F7025 x F4 progeny

A set of 284 RILs was developed at INRA Lusignan (France) by single seed descent in the cross between the medium early dent line F7025 and the early flint line F4. F4 originated from the French variety «Etoile de Normandie» while F7025 was bred in a cross between an Iodent-related line and F113bm3. For hybrid experiments, RILs were topcrossed with the dent line F7038, originating from the backcross of a late hybrid resistant to lodging and stalk breakage (BSSS and Lancaster related) with the early dent line F252 (Co125, Reid Yellow dent, and Minnesota13 related). F7038 cell wall digestibility (IVNDFD) is rather poor, about 3 percentage points lower than the one of F7025.

Field experiments were carried out in 2 locations over 3 years for RIL *per se* values at Lusignan (Vienne, 2001, 02, 03) and Le Pin (Orne, 2001, 02). Field experiments of topcrosses were carried out at Lusignan and Le Pin over 2 years (2002, 03). For both lines and hybrids, genotypes were evaluated in generalized alpha-lattice designs with 2 replicates for the tested RILs, and about 15 replicates of each parents (F4 and F7025 in *per se* experiments, and F4 x F7038 and F7025 x F7038 in topcross experiments). Each experimental plot was a 5.2 m long single row of 37 plants in Lusignan, and a two similar row plot in Le Pin. Row spacing was 0.75 m, and the resulting density was 95,000 plants ha^{-1}. Irrigation was applied in Lusignan during summer to prevent water stress (about 35 mm). The plots were machine-harvested with a forage chopper at an early silage harvest stage except in 2003 at Lusignan with a later stage of harvest after very hot summer conditions. A representative sample of 1 kg chopped material per plot was collected for dry-matter (DM) content estimates and biochemical analyses.

For the linkage map construction, nearly 250 simple sequence repeat (SSR) markers were chosen in the maize database (MaizeDB, www.maizegdb.org) throughout the genome according to their bin location. Out of these markers, 94 giving different banding patterns in the two parental lines F7025 and F4 were successfully used on 231 out of the 284 RILs by Eurofins (Agrogene), after DNA extraction also performed by the same company. The linkage map was developed using Mapmaker software (version 3.0b, Lincoln et al, 1992). Analyses of variance were carried out following the standard procedure of a fixed model with genotype, environment (year-location), block, sub-block and interaction effects. Phenotypic correlations between traits were computed on the mean basis over years and locations. QTL identification was based on means over years and locations of 231 RILs in *per se* and topcross experiments, using the method of Composite Interval Mapping (CIM; Zeng, 1994) implemented in the PLABQTL computer package (Utz and Melchinger, 1995; 1996) as previously described (Barrière et al, 2008). Using the permutation test method of Churchill and Doerge (1994), LOD thresholds equal to respectively 2.9 and 3.7 allowed an experiment-wise error rate respectively close to 5 and 1%, respectively.

QTL physical positions were estimated based on physical position of the two flanking markers, assuming a constant Mbp / cM ratio within these intervals (B73 AGPv4 release, maizesequence database). However, QTL physical positions were less accurately obtained in bins 1.04 and 4.06, as markers and QTLs were in proximity to chromosome centromeres. Genetic positions of centromeres were estimated on the RIL map with similar assumptions as for QTL physical positions.

The list of putative candidate genes, which could underlie cell wall digestibility QTLs, was established from a large synthesis of published investigations devoted to cell wall metabolism and assembly, including genes already known in maize and maize orthologs of rice, Arabidopsis, eucalyptus, poplar, pine, and zinnia genes (Courtial et al, 2013). Although this list is regularly up-dated, it cannot be considered as exhaustive, especially because some grass-specific cell wall genes probably remain still unidentified, as well as roles in cell wall properties of genes with known or still unknown functions are not yet discovered.

Investigations of cell traits in a set of early lines, including F4

A set of early lines including F4, F4 progenies, and brown-midrib bm3 lines was experimented in Lusignan in 2008 and 2009. Field experiment conditions and protocols were similar to those used in RIL *per se* experiments, except lattice design with two replicates instead of alpha-lattice design.

Transcriptomic analysis

The two INRA lines F4 and F2 were cropped in a greenhouse during the spring of 2004 at Lusignan, in pots with a mix of sand and compost, and were fed with a usual nutritive solution. Plants were harvested at early silking, just before pollen shedding. The internode below the node bearing the ear was sampled from three plants. Internodes of grasses elongate in acropetal staggered succession, with an intercalary meristematic zone at the base of each internode which remains active until the final stage of elongation (Cherney et al, 1989; Scobbie et al, 1993). The ear internode is a newly extended one and is fully lignifying at this stage (Morrison and Buxton, 1993; Morrison et al, 1998). Nodes and leaf sheaths were eliminated and ear internodes were immediately frozen in liquid nitrogen after their harvest.

RNA extraction and purification, maize cell wall macro-array construction, macro-array cDNA probe elaboration, membrane hybridization, and data analysis were managed as previously described by Guillaumie et al (2008). The maize cell wall macro-array, which consists of gene specific tags (GSTs) for 651

genes, was also previously described (Guillaumie et al, 2007). Spotted genes were chosen i) as maize known genes related to the lignin pathway, ii) as orthologs of genes expressed during secondary cell wall formation of zinnia tracheary elements (Pesquet al, 2005) and iii) from a keyword strategy developed from sequences described as involved in primary and secondary cell wall biosynthesis and assembly in all plant species. Maize orthologous genes were found from blastn and tblastx searches performed against GenoPlante-Info databases (Samson et al, 1997). Annotations were verified and searches for maize reference gene numbers (maizesequence AGPv4) were done based on blast request on the maize AGPv4 sequence. Only results for genes involved in lignin biosynthesis and deposition, and for a few miscellaneous other genes differentially expressed in internodes of the two F4 and F2 lines, were reported in the search for cell wall specificities of the F4 line.

Lignin histochemical staining

Stem sections were cut with a vibratome. Phloroglucinol reactions were performed according to standard protocols. According to magnification, stalk tissue sections were observed either using a binocular loupe or an inverted microscope (Leitz DMIRBE). Images were registered using a CCD camera (Color Coolview, Photonic Science) and treated by image analysis (Image Pro-Plus, Media Cybernetics).

Results and Discussion

History of the old INRA F4 line

The F4 line has been self-pollinated at INRA Versailles at the end of the 1940 decade in the variety «Etoile de Normandie» and this line was released in 1955. The «Etoile de Normandie» variety was bred and developed by the Agarkoff Company (Supplementary Figure 1), located at Château du Mesnil sur l'Estrée (Eure), in the south eastern of the French Normandy (Jussiaux, 1955).

According to old documents that were kept at INRA Montpellier, the «Etoile de Normandie» variety was likely bred in a cross done in the middle of the 1930 decade between a flint Pomeranian landrace and the male parent of the flint Long Yellow King variety cropped in Canada. In the 1940 decade up until the second half of the 1950 decade, «Etoile de Normandie» was used both for grain [yielding about 4 - 5 t ha^{-1}, with sowing at 40 - 50,000 plants ha^{-1}, about 1 t ha^{-1} less than Wisconsin240, a double-cross (Wisconsin dent x northern flint) hybrid, (WD x W9) x (W85 x W15), introduced in France in 1947] and forage production (yielding about 7 - 8 t ha^{-1}, with sowing at up to 300,000 plants ha^{-1} and harvest just after flowering, about 1.5 t ha^{-1} less than Wisconsin240). At that time, Etoile de Normandie and Wisconsin240, were the only two maize varieties registered on the French list as «very early». The variety INRA200, first double-cross Wisconsin dent x Lacaune flint hybrid (WH x WJ) x (F7 x F2), was registered latter, in 1957 (Supplemental references).

According to the classification done by Rautou (1954) of 120 French maize landraces, «Etoile de Normandie» belongs to the same group as «Jaune d'Alsace» and «Jaune de Bade» (both «Bade Yellow»). This classification was afterwards confirmed based on molecular data, as F4 was shown being at least 95% related to the northern flint group (Gouesnard et al, 2017). This latter fact suggests that the Long Yellow King male genotype also belonged to the American northern flint germplasm. The similarity between American northern flint and European northern flint landraces has been indeed established from molecular investigations showing that flint germplasm of north and eastern Europe directly originated from American northern flint populations (Rebourg et al, 2003; Brandenburg et al, 2017). Northern flint germplasm was likely first introduced in Europe through the French Normandy since the first half of the 16th century, possibly after the travels of Giovanni Verrazano and Jacques Cartier that reached the Saint-Laurent river area. Northern flint maize spreads at once to Germany and northern and eastern Europe, without diversification through crosses with southern introductions.

Cell wall digestibility related traits in the old INRA F4 line

The F4 line is typified by its very high cell degradability in *per se* value, higher to the one of bm3 lines such as F7026bm3 or F2bm3 (Table 1), but F4 does not exhibit a brown-midrib phenotype. Most cell wall components appeared nearly similar in F4 and in other lines of high cell wall digestibility such as F7. In addition to cell wall digestibility, F4 differed from F2 through a significantly lower lignin content and *p*-coumarate release. The F7087 line, which is a progeny of F4 bred for high cell wall digestibility, has similar cell wall traits as F4, but is significantly improved for *per se* whole plant and grain yields, and also standability. As a consequence of the COMT gene disruption in the *bm3* mutants, the biosynthesis of syringyl units is impaired and the S/G (Sg/Va) ratio of bm3 lines is consequently reduced to value close to 0.70, while it usually ranged from 1.10 to 1.25 in normal lines (1.10 in F4). However, while a low S/G ratio was thus observed in F2bm3, F7019bm3, and F7026bm3 lines, the S/G ratio in F4bm3 was equal to 0.96, closer to the one of normal lines and despite the significant increase of cell wall digestibility in F4bm3 in comparison to F4. This less reduced S/G ratio was related to a lower reduction of syringyl units in F4bm3 than in the other considered bm3 lines.

The F7106 line, which was bred at INRA Lusignan in a Netherland flint landrace (VC150, originating from an old landrace of the Dutch Gelderland province), is at least 92% northern flint related and had a nearly similar cell wall digestibility as F4. However, F7106 had higher lignin content than all considered lines of high cell wall digestibility, similar to the one observed

Table 1 - Comparisons of cell wall traits in the F4 line, F4 progeny lines, and a set of lines with cell digestibility higher than the one of the F2 line. F7038 is the tester line in QTL topcross experiments and F874 is a control line of low cell wall digestibility.

Line	Pedigree	IVNDFD	ADL/ NDF	KL/ NDF	pCA	ester FA	ether FA	pHb	Va	Sg	Sg/Va
F4bm3	F4 x bm3	43.8	4.09	12.0	8.6	5.32	1.22	1.01	4.74	4.57	0.96
F7019bm3	F7019 x bm3	41.8	3.66	11.7	9.2	6.39	1.22	1.18	6.04	4.56	0.75
F4	Etoile de Normandie	39.7	4.43	12.0	8.2	5.10	1.27	1.34	5.65	6.20	1.10
F7087	(F324 x F4) x F324	39.6	4.78	13.5	9.7	5.56	1.22	1.48	5.34	5.74	1.07
F7106	Gelderland-VC150	39.4	5.77	14.8	5.7	3.85	1.25	1.46	6.37	5.05	0.79
F2bm3	F2 x bm3	38.5	4.72	13.0	7.5	5.38	1.19	1.17	5.86	4.23	0.72
F7026bm3	Iodent x F113bm3	38.4	4.74	12.9	5.6	5.97	1.24	1.29	6.12	4.06	0.66
F7	Lacaune	37.7	4.85	11.8	7.0	5.00	1.23	1.15	5.60	6.14	1.10
F324	(F282 x F283) x F286	36.7	5.05	15.0	10.5	5.68	1.05	1.52	6.01	6.62	1.10
F7019	((A632 x B59)-97-2) x F113	34.5	4.77	12.3	12.1	6.64	1.30	1.66	7.69	7.86	1.02
F7025	Iodent x F113bm3	32.5	5.37	15.1	13.7	6.33	1.29	1.92	7.70	9.19	1.19
F2	Lacaune	31.6	5.12	14.2	11.1	5.62	1.38	1.52	7.36	8.03	1.09
F7038	(RVD4 x F252) x F252	29.6	5.14	15.2	15.2	6.62	1.34	2.04	8.00	9.75	1.22
F874	Ostrinia tolerance synthetic	23.5	6.11	15.5	14.6	5.82	1.27	1.96	7.71	10.76	1.40
conf limit	-	1.9	0.45	0.83	0.9	0.32	0.11	0.14	0.53	0.80	-

INRA Lusignan experiments, IVNDFD, ADL/NDF, and KL/NDF as percentages, esterified *p*-coumaric, esterified and etherified ferulic acids (pCA, esterFA, and etherFA) as mg g^{-1} NDF, *p*-hydroxybenzaldehyde, vanillin, and syringaldehyde (pHb, Va, Sg) as mg g^{-1} NDF, conf limit - confidence limit

in F2. Conversely, F7106 had the lowest releases of esterified *p*-coumarate and ferulate. This line also had a Sg/Va ratio close to the one of bm3 lines, with a low proportion of syringyl units. The comparison of cell wall traits in F4 and F7106 thus highlighted that different determinants could underlay cell wall digestibility variations, with their possible stacking during breeding programs.

The effect of COMT down-regulation by a COMT antisens event (COMT-AS; Piquemal et al, 2002) has been investigated in seven genetic backgrounds (Pichon et al, 2006), including the F4 line and the two F2 and F7025 lines (Table 2). As it was observed in bm3 plants, no change in esterified and etherified ferulate releases were shown between normal and COMT-AS progenies. All COMT-AS progenies had lower thioacidolysis yields and lower amounts of S units, with correlative lower *p*-coumarate releases, corroborating results previously observed in bm3 and COMT-AS plants (Piquemal et al, 2002; He et al, 2003; Barrière et al, 2004b). However, the proportion of S units was reduced to nearly 65% in F2 and F7025 COMT-AS plants, while it was only reduced to 75% in F4 COMT-AS plants, with a lower decrease of the S/G ratio. In addition, as it was previously observed in bm3 plants, a significant increase in 5-OH-G units was observed in COMT-AS lines, but not in the F4 COMT-AS progeny. The lack of 5-OH-G unit accumulation, which is the signature of COMT deficiency, in F4 COMT-AS progenies strengthened an atypical monolignol biosynthesis in this line. Finally, the COMT-AS did not allowed cell wall digestibility improvement in the F4 progeny, while significant ones were shown in other investigated lines including F2 and F7025, this fact corroborating the atypical secondary wall biosynthesis and assembly in the F4 line.

Complementarily, the *in vivo* cell wall digestibility (NDFD, NDF *in vivo* digestibility) had been estimated for the two hybrids F7026bm3 x F2bm3 and F7026bm3 x F4 with wethers in digestibility crates (INRA Lusignan unpublished data, methodology described in Barrière et al, 2004a). These measurements contributed to establish that F4 is not primarily affected in a COMT deficiency, because the cell wall digestibility was significantly lower in F7026bm3 x F4 than in F7026bm3 x F2bm3, with NDFD values equal to 49.2 and 59.4%, respectively.

Among the cell wall particularities of the F4 line, from histological observations, the lignification of its parenchyma appeared very discreet, and the lignification of its vascular bundles and surrounding areas was also less pronounced than in F2 (and other flint lines, data not shown). These histological traits likely contribute to the higher cell wall digestibility in F4, and probably also correspond to different and/or specific regulations in tissue patterning and secondary wall lignification in F4 (Figures 1 and 2).

QTL investigations in the F7025 x F4 progeny

Average distance between markers was equal to 17.7 cM on the map of the F7025 x F4 RIL progeny. However, four areas remained with markers distant by more 50 cM, two areas on chromosomes 1, one on chromosome 2, and another one on chromosome 10. Two markers, bnlg400 and bnlg1191, were mapped in positions and orders differing from those given in the MaizeGDB database (bnlg400 mapped in bin 1.06 instead of bin 1.09, and bnlg1191 mapped in bin 9.06

Table 2 - Means for cell wall digestibility, biochemical, and thioacidolysis lignin monomeric traits in F4, F2, F2025 normal plants and their COMT-AS progenies, data from Pichon et al (2006).

Line	Normal/ COMT-AS	IVNDFD	ADL/ NDF	KL/ NDF	pCA	ester FA	ether FA	H	G	S	H+ S+G	5-OH-G	S/G
F4	Normal	35.9	5.4	13.3	10.0	5.3	0.9	9.8	286	367	669	6.8	1.29
F4	COMT-AS	35.9	5.3	12.6	9.3	5.1	1.0	7.7	202	272	487	4.4	1.35
F2	Normal	22.8	6.0	14.3	10.8	5.3	1.2	13.0	345	379	744	6.8	1.09
F2	COMT-AS	28.2	5.2	13.2	9.2	5.4	1.1	8.7	246	240	511	17.3	0.98
F7025	Normal	22.6	6.0	15.8	11.7	5.1	1.3	19.3	352	487	866	7.7	1.39
F7025	COMT-AS	30.1	5.0	13.5	9.0	5.2	1.2	10.1	358	328	734	38.3	0.92

IVNDFD, NDF, ADL, and Klason lignin (KL) as percentages; *p*-coumaric acid (pCA), esterified and etherified ferulic acid (esterFA and etherFA) as mg g^{-1} NDF; *p*-Hydroxyphenyl (H), Guaiacyl (G), Syringyl (S), 5-hydroxyguaicyl (5-OH-G) units as μmole g^{-1} Klason lignins

instead of bin 9.07. Moreover, markers bnlg2160, described as bnlg2160a and bnlg2160b in bins 7.01 and 3.05, respectively, were mapped on the F7025 x F4 map in bins 4.06 and 5.02 and named bnlg2160c and bnlg2160d, respectively (Supplementary Figure 2). Ratios between physical and genetic distances between two successive markers ranged between 0.2 and 5.0 Mbp / cM and mainly matched with positions relative to centromeres.

In line *per se* experiments, average DM content at harvest was equal to 29.1% and ranged from 21.3 to 43.1% as means over the five environments (34.1 and 29.3% in F4 and F7025, respectively). In topcross experiments, average DM content at harvest was equal to 37.1% and ranged from 31.0 to 46.9% as means over the four environments (44.8 and 33.0% in F4 x F7038 and F7025 x F7038, respectively). The high DM content values are related to the especially hot and dry conditions of the 2003 summer. In line *per se* experiments, average DM yield at harvest was equal to 6.6 t ha^{-1} and ranged from 2.9 to 11.1 t ha^{-1} (2.6 and 9.6 t ha^{-1} for F4 and F7025, respectively). Average topcross DM yield was equal to 14.7 t ha^{-1} and ranged from 8.1 to 19.6 t ha^{-1} (10.8 and 16.7 t ha^{-1} in the two F4 x F7038 and F7025 x F7038 hybrids, respectively). For investigated cell wall traits (lignin contents and cell wall digestibility), all genotype effects were highly significant, and genotype x location interactions were always lower than genotype effects (Table 3). Correlations between *in vitro* cell wall digestibility and lignin contents were higher with ADL lignins than with Klason lignins, these two lignin estimates explaining nearly 65 and 35% of variation for IVNDFD cell wall digestibility, respectively, in both line *per se* and topcross experiments.

For lignin contents and cell wall digestibility, QTLs decreasing lignin content and increasing cell wall digestibility (favorable QTLs and alleles) were shown originating from the two F4 and F7025 lines, but with a higher frequency of favorable alleles originating from F4. Favorable QTL positions originating from F7025 are probably related to its Minnesota13/Oh43 (F113) origin, rather than to its Iodent lineage. In addition, it cannot be ruled out that some favorable genomic positions of F4 have been lost during RIL production due to possible linkages between favorable cell wall traits and determinants inducing a too low vigor of some progenies.

Ten QTLs positions for the investigated cell wall traits were shown from *per se* and topcross experiments (Table 4). It was considered that the IVNDFD QTL found in bin 1.04 (position 116 cM) likely colocalized with the set of QTLs located a little upstream, its position being probably misestimated due to its centromere proximity. Among the ten QTL positions, two were shown from only *per se* values (QTLs in bins 4.08 and 8.05) with favorable alleles from F7025, and three from only topcross values (QTLs in bins 2.01, 3.02, and 6.07) with favorable alleles from F4. QTLs for IVNDFD with F4 favorable alleles were located in four positions in *per se* value experiments and five positions in topcross experiments, and explained at all 29% (bins 1.03, 2.03, 6.05, and 9.06) and 64% (bins 1.04, 2.01, 2.03, 3.02, and 6.07) of phenotypic variations in line and hybrid experiments, respectively. In addition, a significant epistatic interaction was shown between IVNDFD QTLs located in bins 1.03 and 6.05 with an increased favorable effect on IVNDFD when F4 alleles were present at the two positions (line *per se* experiments). Complementarily, QTLs for IVNDFD with F7025 favorable alleles were located in three and one positions in per se and topcross experiments, and explained at all 30% (bins 4.06, 4.08, and 8.05) and 10% (bin 4.06) of phenotypic variations, respectively. A greater part of phenotypic variations for cell wall digestibility was explained in topcross experiments by favorable alleles originating from F4, a fact that could be related to the high cell wall digestibility of F4 together with the partly common US ancestry of F7025 and the F7038 tester line. However, considering F4 favorable alleles, the higher part of explained variations in topcross than in *per se* experiments was not expected, due to the high cell wall digestibility of F4 in *per se* value. This fact could possibly be explained by the low vigor of «F4-like» RILs that possibly made more difficult the plant growing and further cell wall digestibility estimates. In addition, this could also be related to the great earliness of a part these «F4-like»

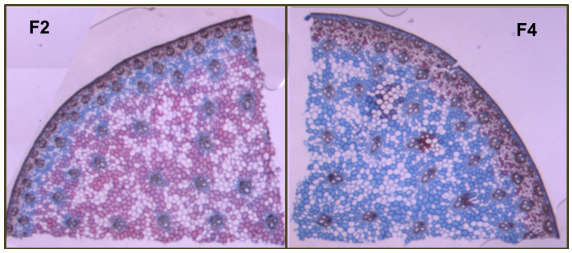

Figure 1 - Histological slides of the F2 and F4 lines (Fasga coloration, lignin and cellulose stained in red and blue, respectively, magnification x 0.8, slides from Méchin, 2000).

RILs, for which a higher DM content at harvest had likely led to underestimate their cell wall digestibility. No colocalization between IVNDFD QTLs and silking date QTLs was nevertheless observed, either in *per se* value or topcross experiments (data not shown).

Considering both line *per se* and topcross experiments, F4 favorable alleles (or determinants) appeared first located in bins 1.03 and 2.03, and then in the five other found QTL positions. The interest of the F4 for silage maize improvement was thus not only related to one or two major traits, but seemed also related to the presence of a few other favorable alleles in the F4 genome, of which number is possibly underestimated. Complementarily, only one major position for favorable cell wall digestibility allele(s) was shown in the F7025 line, in bin 4.06.

Comparison of gene expression in the two F4 and F2 inbred lines

Comparison of cell wall related gene expression in the two F4 and F2 inbred lines

Comparisons of gene expressions in lignifying internodes of the two F4 and F2 lines were first considered for genes involved in monolignol biosynthesis, their polymerization, and then for phenylpropanoid and a few cell wall carbohydrate related genes (Table 5).

Considering all genes involved in monolignol biosynthesis, investigations in F4 and F2 lignifying internodes have especially shown that the expressions of three ZmPAL genes [*ZmPAL* or *ZmPAL3a*, *Zm00001d017274*, bin 5.05, pos 191.42 Mbp), *ZmPAL3e* (*Zm00001d051161*, bin 4.06, pos 146.71 Mbp) which is a gene having 98.2% sequence identity with the ZmPAL gene, and *ZmPAL3b* (*Zm00001d017275*, bin 5.05, pos 191.47 Mbp)] were significantly reduced in F4. Their expressions were thus decreased to 18, 62, and 71% of the expression values observed in the control line F2. The two first ones (*ZmPAL3a* and *ZmPAL3e*) were highly expressed in the F2 line in the below-ear internode at silking stage, while the third one (*ZmPAL3b*) was weakly expressed. The expression of the *ZmPAL2a* gene (*Zm00001d003016*, bin 2.03, pos 29.54 Mbp) was similar in F4 and F2 lines. PAL proteins catalyze the first step of the monolignol pathway, allowing the deamination of phenylalanine into cinnamic acid, but in maize (and grass) PAL enzymes

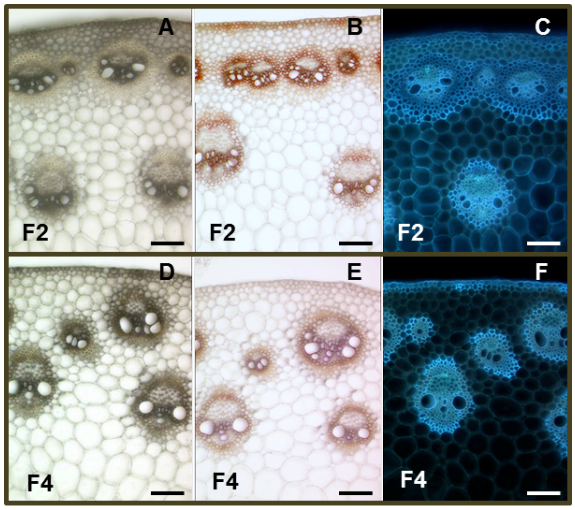

Figure 2 - Histochemical localization of lignin in maize below-ear internodes of the F2 and F4 lines at silking stage (Sections A and D without coloration, white light, sections B and E tissues stained with phloroglucinol reagent, lignin in red, sections C and F, fluorescence of lignified cell wall tissues in response to UV light excitation, magnification bars = 200 μm, slides from Guillaumie, 2006).

also have tyrosine ammonia lyase activity catalyzing deamination of tyrosine into *p*-coumaric acid (Higuchi et al, 1967; Roesler et al, 1997). In Brachypodium, the TAL activity of *BdPTAL1* (*Bradi3g49250*) was shown to provide nearly half of the cell wall lignin with a preference for S units and also of wall-bound *p*-coumarate (Barros et al, 2016). According to the maizesequence database, the *ZmPAL3a* and *ZmPAL3e* are the two orthologs of *BdPTAL1*. The *ZmPAL3* genes are considered as those preferentially involved in the monolignol pathway. Other ZmPAL genes have been shown in the maize genome since the construction of the used macro-array, of which expression is thus not known, including two genes in close positions to the *ZmPAL3a* and *ZmPAL3b* genes [*Zm00001d017276* (ZmPAL3c) and *Zm00001d017279* (*ZmPAL3d*), bin 5.05, pos 191.48 and 191.54 Mbp] and two genes in close positions to *ZmPAL3e* [*Zm00001d051163* (*ZmPAL3f*) and *Zm00001d051166* (*ZmPAL3g*), bin 4.06, pos 146.79 and 146.85 Mbp]. Anyway, *ZmPAL3a* and *ZmPAL3e* have very likely the priority role in monolignol biosynthesis, all the more as these two genes were those that were first evidenced from their mRNA.

Possibly as a consequence of the reduced ZmPAL expression, but possibly also related to a common upstream regulation, expressions of the *ZmF5H1*, *ZmCOMT*, and *ZmCCR1* genes were simultaneously reduced in F4 to 51, 57, and 73% of those of the F2 control line, respectively. The F5H protein catalyzes the hydroxylation of coniferaldehyde into 5-hydroxyconiferaldehyde, which is then methylated into sinapaldehyde in a reaction catalyzed by the COMT. Only the *ZmF5H1* gene was spotted on the cell wall

Table 3 - Variance analysis, mean square, mean, maximum, and minimum values of cell wall related traits for 231 F7025 x F4 RIL progenies in *per se* and topcross experiments (all genotype and genotype x environment mean-squares (MS) were significant at $P < 0.001$, all traits as percentages).

Traits *per se*	genotype MS	gen x env MS	σr^2	mean	mini	maxi	F4	F7025
ADL/NDF	1.9	0.2	0.1	5.2	3.8	6.4	4.8	6.1
KL/NDF	5.9	1.3	0.5	14.4	11.8	17.0	13.6	15.3
IVNDFD	45.9	6.4	3.1	26.8	21.4	34.7	30.3	25.9
Traits topcross	genotype MS	gen x env MS	σr^2	mean	mini	maxi	F4 x F7038	F7025 x F7038
ADL/NDF	0.37	0.10	0.08	6.4	5.5	7.1	5.8	6.7
KL/NDF	1.17	0.40	0.28	13.9	12.7	14.9	13.5	14.2
IVNDFD	7.3	1.8	1.5	25.7	22.6	28.7	27.2	24.6

macro-array, but current results showed that at least two *ZmF5H* genes exist in the maize genome, with likely a primary involvement of the ZmF5H1. The lower ZmF5H1 expression in the F4 line could contribute to explain the weak presence of 5-OH-guaiacyl units in the lignin of F4 COMT-AS plants (Pichon et al, 2006). The *ZmCCR1* gene was significantly more expressed in both F2 and F4 lines than the *ZmCCR2* gene, this latter one being considered to be mostly involved in stress response (Pichon et al, 1998). The tendency to a lower ZmCCR1 expression in F4 lignifying internodes could probably be related to a feed-back effect resulting from the lower availability of coniferaldehyde and sinapaldehyde during monolignol biosynthesis in this line. Moreover, in addition to decreasing lignin content, reduced CCR activity also has as consequence an increase of monolignol ferulate incorporation into maize lignins, introducing more labile linkages in the lignin backbone, thus contributing to cell wall degradability improvement at least after alkaline pretreatment (Ralph et al, 2008; Tamasloukht et al, 2011; Park et al, 2012; Wilkerson et al, 2014; Smith et al, 2017).

C4H enzymes catalyze the conversion of cinnamic acid into *p*-coumaric acid, which appears the main way to *p*-coumaric acid biosynthesis, even if the TAL activity of PAL enzymes allows a direct biosynthesis of *p*-coumaric acid from tyrosine. Whereas *ZmC4H1* expression was not modified in F4 in comparison with F2 lines, *ZmC4H2* was more than five times more expressed in F4, possibly as a feedback effect of the lower availability of cinnamic acid due to reduced ZmPAL activities. However, *ZmC4H1* was nearly three times more expressed than *ZmC4H2* in F2.

At least, two Zm4CL genes are currently considered as involved in maize monolignol biosynthesis, with a likely primary role for *Zm4CL1*. The *Zm4CL1* gene is indeed orthologous to the sorghum Sb04g005210 *Sb4CL* gene of which missense mutations induced the brown-midrib bmr2 phenotype (Saballos et al, 2012). The *Zm4CL1* gene was not differentially expressed between F4 and F2 lignifying internodes. However, the *Zm4CL2* gene, which was similarly expressed as the *Zm4CL1* one in F2, was nearly three times more expressed in F4 than in F2, two facts possibly indicating a significant involvement of the *Zm4CL2* gene in maize monolignol biosynthesis.

CCoAOMT enzymes catalyze the methylation of caffeoyl-CoA into feruloyl-CoA, opening the way to the biosynthesis of coniferyl and syringyl alcohols. Feruloyl-CoA is also involved in the arabinoxylan feruloylation pathway, allowing afterwards cross-linkages between secondary wall components. Even if five ZmCCoAOMT genes were shown in the maize genome, only the two ZmCCoAOMT1 and ZmCCoAOMT2 proteins are considered to have all catalytic sites allowing the enzymatic activity on caffeoyl-CoA (Walker et al, 2016). The two *ZmCCoAOMT2* and *ZmCCoAOMT5* genes were significantly more expressed than the three other ZmCCoAOMT in F2 lignifying internodes, while *ZmCCoAOMT3* and *ZmCCoAOMT4* were more expressed in F4 than in F2. However, the role of these two latter in the monolignol pathway, as well as the role of *ZmCCoAOMT5*, is not established.

Maize *ZmCAD2* and *ZmCAD1* genes are orthologous to eucalyptus *EgCAD2* and *EgCAD1* (Goffner et al, 1992; Goffner et al, 1998), respectively. *EgCAD2* is a zinc-containing long-chain alcohol dehydrogenase active as dimeric form (Jornvall et al, 1987), while the *EgCAD1* gene encodes a short-chain alcohol dehydrogenase active as monomeric form (Hawkins and Boudet, 1994; Jornvall et al, 1995; Boudet et al, 2004). EgCAD2-like proteins are considered as the primarily involved CAD enzymes of the monolignol pathway. Function of EgCAD1-type proteins in the last step of monolignol biosynthesis is not completely elucidated, but *EgCAD1* was shown to be involved in the synthesis of coniferyl alcohol, in addition with *EgCAD2*, likely with different spatio-temporal regulations (Damiani et al, 2005). The two maize *ZmCAD1* and *ZmCAD2* appeared with a tendency to be more expressed in F4 than in F2 lignifying internodes, possibly also as a feedback effect of the lower ZmPAL activity.

One peroxidase (*ZmPox2*) and one laccase (*Zmlac4*, Atlac17-like and Oslac4-like) were significantly

Table 4 - Putative QTLs identified for cell wall lignification traits from 231 RILs in the F7025 x F4 progeny, from line *per se* experiments (line, 3 years and 2 locations) and topcross experiments (topcross, 2 years and 2 locations).

Trait	line/topcross	chr	bin	QTL left marker	QTL pos cM	sup int cM	QTL pos Mbp	sup int Mbp	Lod	R^2	add	line+
ADL/NDF	line	1	1.03	bnlg176	76	68 - 86	51.5	43 - 62	9.5	17.3	0.18	F7025
KL/NDF	line	1	1.03	bnlg176	70	56 - 88	45.3	31 - 64	2.5	4.8	0.19	F7025
IVNDFD	line	1	1.03	bnlg176	78	68 - 86	53.5	43 - 62	5.7	10.8	0.70	F4
ADL/NDF	topcross	1	1.03	bnlg176	72	64 - 90	47.4	39 - 66	7.4	13.8	0.10	F7025
KL/NDF	topcross	1	1.04	bnlg2238	88	82 - 114	73.2	60 - 131	6.6	12.4	0.13	F7025
IVNDFD	topcross	1	1.04	bnlg2295	116	104 - 126	135.0	109 - 157	8.7	16.0	0.58	F4
ADL/NDF	topcross	2	2.01	phi96100	32	18 - 52	8.0	5 - 12	3.7	7.5	0.08	F7025
KL/NDF	topcross	2	2.01	phi96100	20	2 - 36	5.6	2 - 9	3.5	7.0	0.13	F7025
IVNDFD	topcross	2	2.01	phi96100	14	2 - 24	4.4	2 - 6	8.6	16.5	0.43	F4
ADL/NDF	line	2	2.03	bnlg381	102	78 - 110	28.3	21 - 35	3.1	5.9	0.10	F7025
KL/NDF	line	2	2.03	bnlg381	106	88 - 112	32.6	27 - 37	7.1	13.2	0.31	F7025
IVNDFD	line	2	2.03	bnlg2277	90	68 - 114	24.2	18 - 31	2.6	5.0	0.69	F4
KL/NDF	topcross	2	2.03	bnlg2277	88	78 - 108	23.6	21 - 30	8.8	16.2	0.25	F7025
IVNDFD	topcross	2	2.03	bnlg2277	94	78 - 110	25.4	21 - 30	5.3	10.1	0.36	F4
ADL/NDF	topcross	3	3.02	bnlg1144	44	34 - 68	7.6	6 - 13	5.4	10.4	0.08	F7025
KL/NDF	topcross	3	3.02	bnlg1144	34	24 - 46	5.7	4 - 8	3.6	7.1	0.10	F7025
IVNDFD	topcross	3	3.02	bnlg1144	36	22 - 46	6.1	3 - 8	5.5	10.6	0.31	F4
ADL/NDF	line	4	4.06	bnlg252	72	64 - 80	54.6	39 - 71	7.3	13.6	0.16	F4
KL/NDF	line	4	4.06	bnlg252	80	68 - 102	70.5	47 - 115	3.3	6.4	0.23	F4
IVNDFD	line	4	4.06	bnlg252	76	68 - 86	62.5	47 - 83	6.2	11.6	0.84	F7025
KL/NDF	topcross	4	4.06	bnlg2160c	94	80 - 102	98.5	71 - 115	6.4	12.1	0.15	F4
IVNDFD	topcross	4	4.06	bnlg252	78	68 - 86	66.5	47 - 83	5.4	10.3	0.35	F7025
ADL/NDF	line	4	4.08	bnlg2162	128	124 - 142	186.2	183 - 197	3.2	6.2	0.09	F4
KL/NDF	line	4	4.08	bnlg2162	136	124 - 148	192.3	183 - 201	4.2	8.1	0.28	F4
IVNDFD	line	4	4.08	bnlg2162	136	126 - 144	192.3	185 - 198	7.0	12.9	0.94	F7025
ADL/NDF	line	6	6.05	umc1857	92	80 - 102	132.1	115 - 146	5.2	9.8	0.15	F7025
IVNDFD	line	6	6.05	umc1857	92	78 - 116	132.1	113 - 165	3.3	6.3	0.64	F4
ADL/NDF	topcross	6	6.07	bnlg345	158	142 - 172	161.3	158 - 165	3.5	7.1	0.08	F7025
IVNDFD	topcross	6	6.07	bnlg345	158	146 - 172	161.3	158 - 165	5.1	10.3	0.55	F4
ADL/NDF	line	8	8.05	bnlg1782	88	78 - 96	160.2	154 - 165	7.3	13.6	0.17	F4
IVNDFD	line	8	8.05	bnlg1782	96	82 - 114	165.1	157 - 176	3.0	5.9	0.49	F7025
ADL/NDF	line	9	9.06	bnlg1191	124	122 - 134	145.2	144 - 153	6.3	11.8	0.14	F7025
IVNDFD	line	9	9.06	umc1310	126	122 - 140	146.8	144 - 158	3.6	7.0	0.55	F4

Lod >= 5.0, except if colocalizations with other QTLs, line + is line increasing traits, sup int are QTL support intervals, add are QTL estimated additive values.

more expressed in F4 than in F2 lignifying internodes. Peroxidase and laccase belong to multigene families, with likely several of them having specific involvements in monolignol polymerization. The primary role of Atlac17 and one Brachypodium ortholog (*BdLAC5*, Bradi1g66720) in monolignol polymerization has been shown based on mutant investigations (Berthet et al, 2011; Wang et al, 2015). The *ZmPox2* gene only has orthologs in grass species, according to the maizesequence AGPv4 database. Its possible involvement in monolignol polymerization is not definitely established, but in young plantlets, *ZmPox2* was shown to have a similar pattern of mRNA accumulation than *ZmCOMT*. Moreover, *ZmPox2* was localized in vascular tissue (De Obeso et al, 2003).

Two *S-adenosyl-methionine synthetase 3* (*SAM3*) were similarly highly expressed in F4 and F2 lignifying internodes. Methyl transfer reactions are essential in lignin biosynthesis. A SAM synthase was thus one of the most abundant transcripts in maturing sugarcane stems (Casu et al, 2004). The similar expression in F4 and F2 of these two ZmSAM synthases could correspond to the necessity of methyl radicals for OMT activities, even if the *ZmCOMT* gene was less expressed in F4 than in F2 lignifying internodes. However, the two investigated ZmSAM synthases have two paralogs of which expressions have not been investigated (*Zm00001d024754*, bin 10.03, pos 86.46 Mbp, and *Zm00001d009146*, bin 8.03, pos 39.52 Mbp, having 91 and 85% identity with the *Zm00001d010920* gene, respectively). In addition, the specificity of this set of ZmSAM synthases towards the monolignol methylation cycles is not established.

Three ZRP4-like OMT encoding genes were differentially expressed in F4 and F2 lignifying internodes. The SBP1 ZRP4-like OMT was less expressed in F4 than in F2 lignifying internodes. Two other ZRP4-like genes were more expressed in F4 than in F2. The *SBP1* ZRP4-OMT gene corresponds to the SBP1 cDNA first described by Scott-Graig et al (1998) as an herbicide safener-binding protein of maize (SafBP). The possible role of the ZRP4-OMT genes in the monolignol pathway is not known. Contrarily to the COMT, ZRP4-OMT proteins have not all the five regions highly conserved among plant OMT (Ibrahim et al, 1998). However, their involvement in methylation in the monolignol pathway cannot definitely be ruled out, especially because syringyl unit biosynthesis still occurred in maize mutant or transgenic plants deficient in COMT activity. In Arabidopsis, a species without maize ZRP4-like OMT orthologs, S lignins are almost missing in the AtOMT1 mutant (Goujon et al, 2003).

Upstream the monolignol pathway, one chorismate mutase was more expressed in F4 than in F2 lignifying internodes. In the shikimic acid pathway, the chorismate mutase enzyme catalyzes the first committed step towards phenylalanine and tyro-

Table 5 - Maize genes of the monolignol pathway, genes related to the lignin and cell wall carbohydrate metabolisms, and genes of the phenylpropanoid metabolism spotted on the cell wall macro-array.

Gene function	Gene name AGPv4	bin	pos (Mbp)	mRNA	expression F2	expression F4/F2
Phenylalanine / Tyrosine ammonia lyase (MZEPAL, PAL3a)	Zm00001d017274	5.05	191.42	L77912.1	187353	0.18
Phenylalanine / Tyrosine ammonia lyase (ZmPAL3e)	Zm00001d051161	4.06	146.71	L77912.3	207907	0.61
Phenylalanine / Tyrosine ammonia lyase (ZmPAL3b)	Zm00001d017275	5.05	191.47	AY104679	10421	0.72
Phenylalanine / Tyrosine ammonia lyase (ZmPAL2a)	Zm00001d003016	2.03	29.54	CF631905	102659	1.06
Cinnamate 4-hydroxylase (ZmC4H1)	Zm00001d009858	8.03	85.45	AY104175	66372	0.85
Cinnamate 4-hydroxylase (ZmC4H2)	Zm00001d012510	8.08	175.69	CF647652	19988	5.10
4-coumarate coenzyme A ligase (Zm4CL1)	Zm00001d015459	5.04	91.46	AY105108	22522	1.07
4-coumarate coenzyme A ligase (Zm4CL2)	Zm00001d032103	1.07	213.13	AX204867	21414	2.83
Hydroxycinnamoyl-CoA transferase (ZmHCT1)	Zm00001d017186	5.05	188.27	AY109546	11068	0.83
Hydroxycinnamoyl-CoA transferase (ZmHCT2)	Zm00001d003129	2.04	33.33	DR807341	10690	0.76
Coumarate 3-hydroxylase (ZmC3H1)	Zm00001d043174	3.06	190.63	AY107051	10718	1.08
Caffeoyl CoA O-methyltransferase (ZmCCoAOMT1)	Zm00001d036293	6.02	82.19	AJ242980	21976	1.47
Caffeoyl CoA O-methyltransferase (ZmCCoAOMT2)	Zm00001d045206	9.02	16.08	AJ242981	47434	0.96
Caffeoyl CoA O-methyltransferase (ZmCCoAOMT3)	Zm00001d005998	2.07	194.95	AY104670	23740	2.68
Caffeoyl CoA O-methyltransferase (ZmCCoAOMT4)	Zm00001d052842	4.08	202.40	AI855419	13947	2.10
Caffeoyl CoA O-methyltransferase (ZmCCoAOMT5)	Zm00001d052840	4.08	202.40	AY108449	51550	1.32
Cinnamoyl CoA reductase (ZmCCR1)	Zm00001d032152	1.07	214.57	X98083	37894	0.74
Cinnamoyl CoA reductase (ZmCCR2)	Zm00001d019669	7.02	49.32	Y15069	8776	1.51
Ferulate 5-hydroxylase (F5H1)	Zm00001d032468	1.07	227.65	DR966008	45662	0.50
Caffeic acid O-methyltransferase (ZmCOMT)	Zm00001d049541	4.05	33.82	M73235	142203	0.52
Cinnamyl alcohol dehydrogenase (ZmCAD2)	Zm00001d015618	5.04	101.49	Y13733	30285	1.69
Cinnamyl alcohol dehydrogenase (ZmCAD1)	Zm00001d015956	5.04	134.16	AY106077	16082	1.93
Peroxidase (ZmPox39) CWPO-C-like	Zm00001d014467	5.03	49.12	AY106450	19551	0.86
Peroxidase (ZmPox2)	Zm00001d029274	1.03	34.51	AJ401275	10903	2.98
Peroxidase (ZmPox54)	Zm00001d030199	1.05	112.97	AY110228	10476	1.90
Peroxidase (ZmPox3)	Zm00001d037547	6.05	128.95	AJ401276	9180	0.71
Laccase Atlac17-like	Zm00001d042906	3.06	183.58	BG842157	46998	2.20
S-adenosyl-methionine synthetase	Zm00001d010920	8.05	133.48	BT018468	93192	0.76
S-adenosyl-methionine synthetase	Zm00001d040697	3.04	58.92	BG837557	88494	1.19
O-methyltransferase SBP1, ZRP4-like	Zm00001d004689	2.05	131.24	AF033496	23179	0.37
O-methyltransferase ZRP4-like	Zm00001d029359	1.05	67.31	AY105091	14318	2.15
O-methyltransferase ZRP4-like	Zm00001d053156	4.09	216.94	BI245155	9141	2.31
Chorismate mutase	Zm00001d012674	8.08	178.40	AY103806	34104	2.89
Prephenate dehydratase	Zm00001d028712	1.03	44.01	AY109614	19357	1.90
Chalcone synthase	Zm00001d052673	4.08	196.89	AY728476	13474	0.91
Chalcone synthase	Zm00001d007403	2.03	23.095	DT640793	12812	0.53
Chalcone flavanone isomerase AtCHI/TT5-like	Zm00001d034635	1.12	298.58	DV550165	32848	0.48
Arabinofuranosidase AtARAF1-like	Zm00001d044648	3.09	233.94	AY106254	17188	0.50
β-glucosidase AtβGLU40	Zm00001d028199	1.02	26.00	CA403210	25130	1.36
β-glucosidase AtβGLU42-like	Zm00001d042507	3.06	170.10	AY111218	15614	1.96
β-glucosidase AtβGLU43/44-like	Zm00001d033649	1.09	269.62	DN205450	17689	1.98
β-glucosidase AtβGLU43/44-like	Zm00001d033650	1.09	269.67	BG320059	10008	1.41
β-glucosidase AtβGLU43/44-like	Zm00001d033651	1.09	269.67	BQ279747	10385	0.62
β-glucosidase AtβGLU43/44-like	Zm00001d022367	7.05	176.19	AY106991	7447	0.87
β-glucosidase AtβGLU45/46/47-like	Zm00001d025846	10.05	131.48	BG319924	15245	1.58
β-glucosidase AtβGLU45/46/47-like	Zm00001d003044	2.04	30.14	DR814197	8651	0.74

Genes expression in F2 lignifying internodes and comparative expression in F4 and F2 (normalized expression values are given for the F2 line and F4 values are expressed as ratios of signal intensity compared F2. Genes were considered to be significantly differentially expressed when expression ratio values were lower than 0.5 or higher than 2.0)

sine biosynthesis (Mobley et al, 1999; Ehlting et al, 2005), allowing the intra-molecular rearrangement of the enolpyruvyl chain of chorismate to produce prephenate. A chorismate mutase / prephenate dehydratase genes was also more expressed in F4 internodes than in F2. The higher expression of a prephenate dehydratase gene in F4 could be considered in agreement with the probable higher prephenate production due to the greater expression of the chorismate mutase gene. The greater expression of a chorismate mutase gene could possibly be considered as a feedback effect of a lower content of *p*-coumaric acid downstream the reduced ZmPAL activity.

Two chalcone synthase and one chalcone flavanone isomerase genes were less expressed in F4 than in F2 lignifying internodes. Chalcone synthase catalyzes the first step of the flavonoid pathway, condensing a *p*-coumaryl-CoA with three malonyl-CoA into chalcone. The chalcone flavanone isomerase catalyzes the conversion of chalcone into naringenin, another flavonoid. These two genes belong to the phenylpropanoid pathway but not to the monolignol pathway. Their lower expressions in F4 could be related to the reduced PAL activity and consequently reduced *p*-coumaric acid and *p*-coumaroyl-CoA availability occurring also for flavonoid biosynthesis.

The arabinofuranosidase gene less expressed in F4 than in F2 internodes is orthologous to the *AtARAF1* gene encoding a protein belonging to family 51 of glycoside hydrolases, likely involved in cell wall modification. ARAF1 proteins have thus been localized in stem vascular system and plants overexpressing ARAF1 exhibited altered stem architecture (Chavez-Montes et al, 2008). The role of this arabinofuranosidase in grasses is not known, all the more as it was considered to have arabinan-containing pectins as substrates in Arabidopsis.

Eight ESTs corresponding to β-glucosidase

Table 6 - Miscellaneous maize genes spotted on the cell wall macro-array and differentially expressed in F2 and F4 lignifying internodes. Gene expression in F2 and comparative expression in F4 and F2.

Gene function	Gene name AGPv4	bin	pos (Mbp)	mRNA	expression F2	expression F4/F2
Argonaute ZmAGO1e AtAGO1-like	Zm00001d011096	8.05	138.82	AY110984	10027	2.39
HD-zip III IFL1/REVOLUTA-like, ZmRLD1 paralog	Zm00001d032681	1.07	234.07	CO529337	14535	2.30
HD-zip III ATHB9/14-like	Zm00001d033246	1.09	255.87	AI691669	28762	0.44
Zinc finger C3HC4- RING-type	Zm00001d027649	1.01	10.11	AY365035	12074	1.97
MADS-box ZmZAG5 AtAGL6/13-like	Zm00001d051465	4.06	159.61	L46398	15008	2.79
ABC transporter AtABC G-family-member3-like	Zm00001d034918	1.12	305.58	DT653269	13193	2.78
Glutathione S-transferase (ZmGST17, Bronze2-like)	Zm00001d016860	5.05	178.50	AF244682	16243	3.09
Glutathione S-transferase (ZmGST22, Bronze2-like)	Zm00001d024963	10.04	96.97	AF244687	20836	2.62
β-D-glucosidase ZmGLU1 benzoxazinoid-related	Zm00001d023994	10.03	35.17	U44773	67538	2.04
Arabinogalactan protein (AGP ZmRCP1)	Zm00001d041984	3.05	146.59	AB021175	20741	3.45
Arabinogalactan protein (AGP ZmRCP2)	Zm00001d052527	4.08	192.22	AB021176	17816	3.16
Gene of unknown function (Zinnia DV017507 ortholog)	Zm00001d004700	2.05	132.14	AY108760	204322	0.41
Gene of unknown function MtN21 nodulin-like protein	Zm00001d027682	1.01	11.07	CO529888	11372	2.04
Gene of unknown function	Zm00001d020402	7.02	111.55	AY104431	17398	3.32
Gene of unknown function	Zm00001d041173	3.04	102.06	AY106297	19480	2.68
Gene of unknown function	Zm00001d041776	3.05	137.25	CF003946	33399	2.30
Gene of unknown function	Zm00001d024000	10.03	35.68	BG321233	12239	1.90

Normalized expression values are given for the F2 line and F4 values are expressed as ratios of signal intensity compared to F2. Genes were considered to be significantly differentially expressed when expression ratio values were lower than 0.5 or higher than 2.0).

genes involved in carbohydrate metabolic processes were available on the macro-array. Only two maize β-glucosidase genes were more expressed in F4 than in F2 internodes, orthologous to *AtβGLU42* and *AtβGLU43/44*, respectively. According to the TAIR database, *AtβGLU44* is located in the cell wall and *AtβGLU42* is involved in several processes, including cellulose catabolism. In addition, according similarly to the TAIR database, the two *AtβGLU45/46/47* ortholog β-glucosidase genes encode proteins which are involved in cell wall carbohydrate metabolism related to secondary wall biosynthetic processes, but they were not differentially expressed in F4 and F2 internodes.

Miscellaneous genes differentially expressed in lignifying internodes of the two F4 and F2 lines

In addition to genes related to the phenylpropanoid metabolism, a set of genes with probes spotted on the macro-array were shown differentially expressed in F4 and F2 lignifying internodes (Table 6). The involvement of these genes in secondary wall assembly is not always established, as well as some of them are still of unknown functions.

Argonaute genes are involved in the regulation of gene expression via the RNAi Silencing Complex (RISC). Argonaute-like proteins bind miRNA and cleave the target (Kidner and Martienssen, 2005). Based on the maizesequence database and investigations on AGO1-like genes in maize (Qian et al, 2011; Zhai et al, 2014; Xu et al, 2016), five AtAGO1 orthologs can be considered in maize [*ZmAGO1a* (*Zm00001d035747*, bin 6.01, pos 44.78 Mbp), *ZmAGO1b* (*Zm00001d026111*, bin 10.06, pos 138.70 Mbp), *ZmAGO1c* (*Zm00001d002650*, bin 2.02, pos 18.18 Mbp), *ZmAGO1d* (*Zm00001d014875*, bin 5.03, pos 66.76 Mbp), and *ZmAGO1e* (*Zm00001d011096*, bin 8.05, pos 138.82 Mbp)]. The maize Argonaute *ZmAGO1e* gene, which is a close ortholog of *ZmAGO1a*, was more expressed in F4 internodes, possibly indicating a higher repression of the target gene(s) in this line. Arabidopsis *AtAGO1* mutants have been shown defective in post-transcriptional gene silencing and have pleiotropic developmental and morphological defects (TAIR database). The maize considered ortholog could thus only have a distant upstream role in secondary wall assembly.

Several homeodomain-leucine zipper (HD-zip) transcription factors are expressed during plant tissue formation. In Arabidopsis, the IFL1/REVOLUTA HD-ZIP III is involved in cell differentiation, determination of bilateral symmetry, meristem initiation, xylem and phloem pattern formation, and xylem development (TAIR database). It was shown that a heterodimer of the BLH6 protein and the KNAT7 transcription factor binds to the IFL1/REV promoter and thus represses commitment to secondary cell wall biosynthesis in interfascicular fibers (Liu et al, 2014). The maize rolled leaf1 gene (*ZmRLD1*, *Zm00001d048527*, bin 9.07, pos 157.82 Mbp), which also encodes a HD-ZIP III transcription factor, is orthologous to IFL1/REV of Arabidopsis (Zhong and Ye, 1999). The expression of ZmRLD1 is mediated by the Zmmir166 miRNA that was shown to accumulate in phloem (Juarez et al, 2004). On the abaxial side of the leaf, less abundant sclerenchyma was found in *ZmRLD1* mutant than in normal plants, with consequences on mechanical tissue properties (Hay et al, 2000). The IFL1 ortholog more expressed in F4 internodes is the second paralog of *ZmRLD1* (78% identity). Whether the higher expression of this *ZmRLD1* paralog in F4 is related to its lower lignification is not known. Moreover, the ATHB9 (or PHAVOLUTA) and ATHB14 (PHABULOSA) Arabidopsis HDZIP III, of which one ortholog is less expressed in F4 than in F2 internodes, both have overlapping function with IFL1 (TAIR database).

Like other zinc-finger proteins, C3HC4-RING-type proteins have significant roles during plant growth and tissue assembly (Ma et al, 2009). Several members of the zinc-finger gene family have been shown to be expressed in lignifying tissues of Arabidopsis

and eucalyptus, with still unidentified precise functions. Moreover, colocalizations with cell wall related QTLs of C3HC4 zinc-fingers have been shown in Arabidopsis (Chavigneau et al, 2012) and maize (Barrière et al, 2016) RIL progenies. One C3HC4 zinc-finger gene was thus shown more expressed in F4 than in F2 lignifying internodes, with a possible involvement in regulation of secondary wall lignification.

The maize ZmZAG5 MADS-box transcription factor is orthologous to AGAMOUS (*AGL6* and *AGL13*) Arabidopsis genes, which are involved in the regulation of male and female gametophyte morphogenesis (Mena et al, 1995). *ZmZAG5* seemed similarly involved in floral development, but conversely to its close paralog *ZmZAG3*, *ZmZAG5* had a temporally more restricted expression and much lower transcript abundance in floral organs (Mena et al, 1995). Other MADS-box transcription factors, with other maize orthologs, have been related to lignification processes in Arabidopsis. The *SHP1* (or *AGL1*) gene is required, with *SHP2*, for fruit dehiscence. *SHP1* and *SHP2* are both considered to specify the lignified valve margin of mature Arabidopsis siliques (Liljegren et al, 2000). The *AGL15* gene has been shown to be a negative regulator of the AtPRX17 peroxidase (Cosio et al, 2017). The significant expression of *ZmZAG5* in F4 and F2 lignifying internodes at silking stage, higher in F4 than in F2, could correspond to the involvement in regulation of floral development of this transcription factor. *ZmZAG5* cannot currently be clearly related to secondary wall lignification in maize.

ABC transporters constitute a family of proteins found in a large range of organisms and involved in the transport of a broad range of substances and substrates across membranes. ABC transporters were thus supposed or shown to be involved in the secretion of monolignols in the cell walls (Samuels et al, 2002; Paux et al, 2004; Elhting et al, 2005). One ABC transporter was shown more expressed in F4 than in F2 internodes. Its role in monolignol transport has to be established.

Two bronze2-like ortholog genes were more expressed in F4 than in F2 ear internodes. The maize *Bz2* gene encodes a glutathione S-transferase (GST) which is a carrier protein allowing the delivery of anthocyanin cargo from the site of biosynthesis to the tonoplast membrane where an appropriate ABC-type transporter then moves the pigment into the vacuole (Pairoba and Walbot, 2003). It is likely that these differential expressions of bronze orthologs are not related to secondary wall specificities of F4 and F2 lines.

More expressed in F4 than in F2 internodes, the β-glucosidase ZmGLU1 is the 4-hydroxy-7-methoxy-3-oxo-3,4-dihydro-2H-1,4-benzoxazin-2-yl glucoside β-D-glucosidase 1. Benzoxazinoids are a group of indole-derived compounds widespread in grasses (no *ZmGLU1* orthologs in Arabidopsis or Medicago), which have several defense roles including insecticidal, antimicrobial and antifeedant activities. Benzoxazinones are considered to be stored as glucosides and their hydrolysis by β-glucosidases increases their reactivity and toxicity (Wouters et al, 2016). The reglucosylation of DIMBOA benzoxazinoid has been shown to be a strategy of detoxification by maize pest caterpillars (Wouters et al, 2014). In addition to its role on defense compounds, *ZmGLU1* is also involved in others functions such as ABA metabolism, hydrolysis of conjugated gibberellins, and conversion of storage forms of cytokinins to their active forms (maizesequence and UniProt databases). The higher expression of *ZmGLU1* in F4 internodes could not be clearly related to secondary wall assembly.

Arabinogalactan proteins (AGP) have been implicated in various plant growth and developmental processes, and several have been shown to have roles during plant xylogenesis (Schultz et al, 1998; Whetten et al, 2001). However, *ZmRCP1* and *ZmRCP2*, which were first described in the outermost cells of plant root cap (Matsuyama et al, 1999), are distant proteins from those found involved in xylogenesis. The Arabidopsis ortholog of *ZmRCP1* and *ZmRCP2* is a late embryogenesis abundant protein (LEA, *At3g19430*). If many LEA proteins are induced by stresses, some can also be expressed constitutively (Wise and Tunnacliffe, 2004). In sweet potato, lignin content was increased in IbLEA14-overexpressing calli in which an increased expression of several genes involved in monolignol biosynthesis was observed (Park et al, 2011). However, *ZmRCP1* and *ZmRCP2* are not orthologous to IbLEA14. Functions of *ZmRCP1* and *ZmRCP2* are still unknown, and their higher expression in F4 than in F2 internodes could be related to different developmental process in the two lines, as well as a role in maize cell wall biosynthesis cannot be definitely ruled out.

One maize gene, orthologous to an EST shown from the zinnia xylogenesis strategy and expressed during late tracheary element differentiation processes, was less expressed in F4 than in F2 internodes. This maize gene could thus be assumed to be involved in maize secondary wall assembly. A gene of unknown function orthologous to several Medicago truncatula paralog genes annoted as encoding either MtN21 nodulin family protein / EamA-like transporter family protein or auxin-induced 5NG4-like protein was shown more expressed in F4 than in F2 internodes. In addition, a gene of unknown function (*Zm00001d020402*), without ortholog in other grasses, and three paralog genes of similarly unknown functions (*Zm00001d041173*, *Zm00001d041776*, and *Zm00001d024000*), without Arabidopsis and Medicago orthologs, were more expressed in F4 than in F2 internodes. For this latter set of genes, it is currently not possible to draw any conclusion on their involvement or not in lignified tissue biosynthesis or patterning.

Colocalizations of cell wall related QTLs with can-

didate genes

Colocalizations with candidate genes were searched for the seven QTL positions in the F7025 x F4 progeny with favorable alleles (or determinants, decreasing lignin content and increasing cell wall digestibility) originating from F4, including the two strongest QTL positions in bins 1.03 and 2.03. Colocalizations were simultaneously considered for the three QTL positions with favorable alleles originating from F7025 (Table 7).

Colocalizations with ZmMYB transcription factors

MYB genes are transcription factors involved in numerous regulations of diverse plant metabolisms. Several investigations have especially shown the major role of several MYB transcription factors in coordinated regulations of cell wall phenolic and carbohydrate compound biosynthesis, deposition, and organization (Zhong et al, 2008; Zhong and Ye, 2009; Zhong et al, 2011). According to their functions, MYB genes of several species including maize have been classified in different groups by Du et al (2012), pointing out groups related to secondary wall assembly including especially groups G3 «lignin biosynthesis», G4 «phenylpropanoid pathway», G8 «lignin deposition», G21 «development and cell wall thickening», and G30 «cell wall thickening». Colocalizations between ZmMYB and QTLs related to lignification and cell wall digestibility have afterwards highlighted the highly probable roles in secondary wall assembly of ZmMYB classified in other groups, especially ZmMYB of the G13 and G31 «metabolism» groups (Barrière et al, 2015; Barrière et al, 2016).

The set of strong QTLs, which were shown in bin 1.03 for both line *per se* and topcross experiments and gathered lignin content and cell wall degradability QTLs (F4 favorable allele), colocalized with the *ZmMYB019* transcription factor (*Zm00001d029293*). According to the AGPv4 release of the maize genome, this gene is now considered as maize specific. However, it was gathered with MYBs of the G13 group by Du et al (2012) and has to be considered as ortholog of members of this group. The G13 group of MYB genes thus included the *AtMYB61* transcription factor that was shown to participate in regulation of Arabidopsis lignin biosynthesis (Newman et al, 2004) and the *HvMYB33* transcription factor that was shown to be expressed in lignifying tissue of barley (Wissenbach et al, 1993). Moreover, the *PpMYB8* gene, which also belonged to the G13 group, was shown to regulate PAL gene expression as a positive regulator in maritime pine (Craven-Bartle et al, 2013). In addition, *PpMYB8* was shown to bind a well-conserved eight nucleotide long AC-II element of the PAL genes and thus to act as an activator of gene expression. Because three *ZmPAL3* genes were less expressed in F4 than in F2 internodes, it is therefore possible to hypothesize that the *ZmMYB019* transcription factor could be the underlying determinant of this set of QTLs located in bin 1.03, and that its modified expression and/or altered sequence could significantly contribute to explain the lower *ZmPAL3* gene expression and the correlative lower lignification and higher cell wall digestibility of the F4 line.

Corroborating the probable role of ZmMYB transcription factors as explaining determinants of variation of secondary wall traits, the *ZmMYB092* (G8 group, *Zm00001d037334*) and little downstream the *ZmMYB094* (G4 group, *Zm00001d038338*) colocalized with QTLs located in bin 6.05 (F4 favorable allele). MYBs of the G4 group «phenylpropanoid pathway» (EgMYB1 and AtMYB4 orthologs) act as negative regulators while MYBs of the G8 group «lignin deposition» (*AtMYB85* ortholog) act as positive regulators. In bin 2.01, the *ZmMYB026* (*Zm00001d002476*) transcription factor belonging to the G3 group «lignin biosynthesis» is located a very little downstream QTL position and would not be ruled out as a possible QTL underlying determinant. In bin 6.07 (F4 favorable allele), a little downstream QTL position is located the *ZmMYB100* transcription factor (*Zm00001d038878*). The *ZmMYB100* belongs to the G13 «metabolism» MYB group of which several members are involved in regulation of the secondary wall biosynthesis. *ZmMYB100* is also orthologous to *AtMYB85* ranged in the G8 group «lignin deposition» and could be considered as a possible underlying determinant at this position. In bin 8.05 (F7025 favorable allele), the two *ZmMYB117* and *ZmMYB131* (G13 and G4 groups, *Zm00001d011669* and *Zm00001d012255*, respectively) are closely located upstream and downstream QTL positions. Any of these two ZmMYB genes could correspond to the underlying determinant at this QTL position, but a false position could also be hypothesized, with to two close QTL positions corresponding to each ZmMYB protein activity.

Colocalizations with ZmNAC transcription factors

In addition to their involvement in regulation of numerous plant metabolisms, several investigations mostly done in dicotyledons have shown the major role of NAC transcription factors acting as upstream regulators during the secondary wall biosynthesis and assembly (Zhong and Ye, 2008). Moreover, seven maize Secondary Wall NAC (ZmSWN) transcription factors were later shown to be «master transcriptional activator of the secondary wall biosynthetic program» (Zhong et al, 2011).

In bin 2.03 (F4 favorable allele), several strong lignin and cell wall digestibility QTLs colocalized in close positions with the maize NAC *ZmSWN6* and *ZmSWN4* transcription factors (*Zm00001d002828* and *Zm00001d002934*, respectively). In addition, the SNBE (Secondary wall NAC-Binding Elements) site of the *ZmMYB46* gene was shown only activated by the *ZmSWN6* transcription factor (Zhong et al, 2011). *ZmMYB46* (also named *ZmMYB146*, G31 group, *Zm00001d023931*, bin 10.03, pos 29.36 Mbp) is the only maize ortholog of *AtMYB46* and *EgMYB2* which were also shown as master transcription fac-

Table 7 - Summary of QTL positions and colocalizations with candidate genes identified as involved in secondary wall biosynthesis.

Traits	line/topcross	fav line	bin	QTL pos Mbp	sup int Mbp	R^2	Candidate gene	Gene pos Mbp
Lignin/IVNDFD	line/topcross	F4	1.03	54.2	31 - 131	11.8	ZmMYB019 (G13)	65.08
Lignin/IVNDFD	line/topcross	F4	2.03	26.8	18 - 37	10.3	NAC ZmSWN6	24.00
							ZmSWN4	27.12
Lignin/IVNDFD	line	F4	6.05	132.1	113 - 165	8.1	ZmMYB092 (G8)	121.92
							ZmBAHD-III	132.05
							ZmMYB094 (G4)	154.81
Lignin/IVNDFD	line	F4	9.06	146.0	144 - 158	9.4	ZmHMT1	149.09
							ZmRLD1	157.82
Lignin/IVNDFD	topcross	F4	2.01	6.0	2 - 12	10.3	bm6	3.50
							ZmMYB026 (G3)	13.59
Lignin/IVNDFD	topcross	F4	3.02	6.1	3 - 13	9.4	ZmBAHD-IV	7.48
Lignin/IVNDFD	topcross	F4	6.07	161.3	158 - 165	8.7	ZmC3H2	159.79
							ZmMYB100 (G13)	166.30
Lignin/IVNDFD	line/topcross	F7025	4.06	70.5	39 - 115	10.8	NAC SWN3/4-like	61.71
Lignin/IVNDFD	line	F7025	4.08	190.3	183 - 201	9.1	ZmRCP2	192.22
Lignin/IVNDFD	line	F7025	8.05	162.7	154 - 176	9.8	ZmMYB117 (G3)	158.17
							ZmMYB131 (G4)	171.09
							ZmC4H2	175.69

fav line is favorable line with alleles decreasing lignin content and increasing cell wall digestibility, IVNDFD QTL located in bin 1.04, pos 135.0 Mbp, not considered

tors regulating as activators the secondary wall biosynthesis (Goicoechea et al, 2005; Zhong et al, 2007; Zhong and Ye, 2012). As it was considered for the *ZmMYB019* transcription factor in bin 1.03, the *ZmSWN6* transcription factor could be a highly plausible underlying determinant of the set of QTLs located in bin 2.03, all the more as *ZmSWN6* interacts with *ZmMYB46*. A modified expression and/or altered sequence of *ZmSWN6* could thus contribute to explain the lower lignification and higher cell wall digestibility of the F4 line and could also be related to the histological differences between the two lines.

In bin 4.06 (F7025 favorable allele), QTLs colocalized with a ZmNAC transcription factor (*Zm00001d050039*) paralog of the two *ZmSWN3* and *ZmSWN4* NAC transcription factors. All thrice ZmNAC are orthologous to the Arabidopsis NAC VND7 (Vascular Related NAC-Domain 7) which is involved in xylem formation and regulates secondary wall biosynthesis in vessels (Zhong et al, 2008; Yamaguchi et al, 2010).

Colocalizations with genes of the monolignol pathway

QTLs located in bin 6.07 (F4 favorable allele) first colocalized with the *ZmC3H2* gene (*Zm00001d038555*), encoding one of the two *p*-coumaroyl-shikimate/quinate 3-hydroxylase of the monolignol pathway, and secondly with the *ZmMYB100* which was located a little outside QTL support intervals. In bin 8.05 (F7025 favorable allele), the two *ZmMYB117* and *ZmMYB131* (G13 and G4 groups) are the strongest candidates. However, the *ZmC4H2* cinnamate 4-hydroxylase gene (*Zm00001d012510*), which is located at the very downstream position of QTL support intervals, is a possible candidate because this gene was shown greatly more expressed in F4 than in F2 internodes, a fact that would be in agreement with the increasing effect on lignification of the F4 allele at this QTL position. The *ZmC4H1* gene was nevertheless more expressed in F2 lignifying internodes than the *ZmC4H2* gene, possibly indicating a less important role of *ZmC4H2* than *ZmC4H1* in constitutive lignification (but the *ZmC4H1* gene was similarly expressed in F2 and F4 internodes).

Colocalizations with genes involved in arabinoxylan feruloylation

Cross-linkages through ferulate and diferulate bridges greatly impede cell wall carbohydrate degradation and their inhibitory effects on cell wall fermentation have been estimated to be nearly equal to the one of lignins (Grabber et al, 2009). All available results strengthen a feruloyl-CoA origin of cell wall linked ferulates and that the ferulate transfer to arabinoxylans or UDP-arabinoses is catalyzed by an enzyme (or enzymes) encoded by members(s) of the BAHD family (clade A). In rice, down-regulation of such BAHD genes was associated with a 19% reduced ferulate release from leaves of deregulated plants relative to the control (Piston et al, 2010). In Brachypodium, RNAi lines under- and over-expressing the BAHD *BdAT1* (*Bradi2g43520*) gene showed decreased (up to 35%) and increased (up to 47%) releases of ferulate monomers and dimers from stem tissues, respectively (Buanafina et al, 2016). Considering expression in maize stems and in the ferulate-rich pericarp tissue, five members of the BAHD family have been considered in maize as strong candidates for arabinoxylan feruloylation (Chateigner-Boutin et al, 2016). The BAHD of the subgroup IV (*Zm00001d039535*, *ZmBAHD-IV*), which was considered as a priority candidate for arabinoxylan feruloylation, colocalized with QTLs located in bin 3.02, and one of the two BAHD of the subgroup III (*Zm00001d037619*, *ZmBAHD-III*) colocalized with QTLs located in bin 6.05 (with F4 favorable alleles in both positions).

The favorable epistatic interaction between

IVNDFD QTLs located in bins 1.03 and 6.05 could be related to favorable cumulative effects of colocalizing ZmMYB F4 alleles (*ZmMYB019* and *ZmMYB092*, positive regulators, or less probably *ZmMYB019* and *ZmMYB094*, positive and negative regulators). However, a favorable combination between the F4 alleles of the *ZmMYB019* and *ZmBAHD-III* genes should be considered as a more probable hypothesis. The *ZmBAHD-III* genes closely colocalized with the QTL position in bin 6.05. Moreover, strengthening this latter hypothesis, the QTL epistatic interaction, which was observed for the IVNDFD trait, but not for the lignin trait, could then be assumed as a simultaneous reduction of both lignin content and ferulate cross-linkages.

Colocalizations with miscellaneous genes

In bin 2.01 (F4 favorable allele), the *bm6* mutation is located close the QTL position, in addition to the *ZmMYB026* gene located a little downstream QTL position. This brown-midrib mutation is not fully characterized, but it has been mapped to a 180 kb long sequence located in position 3.50 Mbp of chromosome 2, in which ten genes were found (Chen et al, 2012). Maize bm6 plants have lignin content reduced by almost 10% and cell wall digestibility appears about similarly increased as in *bm3* mutant plants (INRA Lusignan unpublished data). A genetic variation at the *bm6* locus of the F4 line could thus be considered, likely less severe than in *bm6* mutant plants as normal F4 plants do not exhibited a brown-midrib phenotype. However, F4bm3 plants were shown to exhibit only very discrete brown-midrib coloration (INRA Lusignan unpublished data). A bm6-like mutation in F4 should thus be ruled out only after at least an allelism test with the *bm6* mutant.

In bin 4.08 (F7025 favorable allele), QTLs colocalized with the arabinogalactan LEA *ZmRCP2* gene (*Zm00001d052527*) which was shown with ZmRCP1, located in bin 3.05, more expressed in F4 than in F2 internodes. However, *ZmRCP2* seems to be a weak candidate at this QTL position where no other gene with an established or probable role in maize secondary wall biosynthesis nevertheless colocalized. Another still unknown determinant could thus be present in this location.

QTLs located in bin 9.06 (F4 favorable allele) first colocalized with the homocysteine S-methyltransferase *ZmHMT1* gene (*Zm00001d048060*). Homocysteine S-methyltransferases belong to the set of inter-dependent methionine-related cycles allowing the supply of methyl groups to SAM-dependent O-methyltransferases (SAM, S-adenosyl-L-methionine) including COMT and CCoAOMT enzymes in the lignin pathway. The methylenetetrahydrofolate reductase (*ZmMTHFR*, *Zm00001d034602*, bin 1.11, pos 297.61 Mbp) also belongs to these methionine dependent cycles and this *ZmMTHFR* gene is altered in the *bm2* mutation (Tang et al, 2014), with significant consequences on cell wall traits and plant vigor. The *ZmHMT1* gene as candidate underlying QTLs at this position cannot be completely ruled out. The *ZmRLD1* gene (*Zm00001d048527*) also colocalized with QTLs in bin 9.06, but was located at the downstream positions of QTL support intervals. In addition a paralog of *ZmRLD1* located in bin 1.07 was more expressed in F4 than in F2 internodes.

Conclusions

Observed differences in gene expressions between F4 and F2 lignifying internodes could be partly related to their different genetic backgrounds and thus not directly explained their different secondary wall patterns. However, the lower expression of three *ZmPAL3*, which are entry genes of the monolignol pathway, and more especially the much reduced expression of the «ZmPAL» gene, is likely a significant determinant of the F4 lower lignification and higher cell wall digestibility. The lower expressions of the *ZmF5H1* and *ZmCOMT* genes probably strengthen the reduced lignification induced by the reduced ZmPAL activity. The simultaneous differential expressions of several genes of the monolignol and lignin pathways in F4 and F2 lignifying internodes, together with the unusually high cell wall digestibility of F4, are almost certainly a consequence of differential upstream regulations. The two stronger QTLs with F4 as favorable alleles colocalized with the *ZmSWN6* and *ZmMYB019* transcription factors. *ZmSWN6* is a master regulator of secondary wall assembly and lignification and *ZmMYB019* belong to the MYB G13 groups of which several members were shown involved in secondary wall biosynthesis and lignification.

Complementary, three other QTLs with F4 favorable alleles colocalized with ZmMYB of the G3, G4, and G8 groups. Major lignin-related ZmMYB could primarily belong in grasses to the G3 (AtMYB58/63-like «lignin biosynthesis»), G4 (EgMYB1/AtMYB4-like, «phenylpropanoid pathway»), and G8 (AtMYB85-like, «lignin deposition») groups, with members of the G13 (AtMYB61-like, «metabolism"). A comparative analysis of R2R3 MYB transcription factors in grasses (rice, maize, and switchgrass) and dicotyledon plants (Arabidopsis and poplar) has indeed shown that the G4, G8, and G13 were grass-expanded groups (Zhao and Bartley 2014). The latter fact strengthens their possible strategic roles as determinants of the high cell wall digestibility and low lignification of the F4 line. Moreover, colocalizations between QTLs with F7025 favorable alleles and ZmMYB and ZmNAC genes strengthened the fact that transcription factors are probably in many cases the underlying determinants of variations in maize lignin content, lignin structure, cell wall components cross-linkages, and secondary wall digestibility. In addition, the fact that favorable alleles also originate from F7025, despite the very high cell wall digestibility in F4, shows that a large diversity of genes are involved in cell wall digestibility variation of which favorable corresponding alleles

can be used in breeding programs.

While it was not investigated in RIL experiments, line comparison had shown that the release of esterified ferulate was lower in F4 than in F7025 (and F2). This result is in agreement with the colocalizations of two BAHD feruloyl transferases with cell wall digestibility QTLs (F4 favorable alleles). Moreover, the favorable epistatic interaction between IVNDFD QTLs located in bins 1.03 and 6.05 could correspond to a favorable combination of *ZmMYB019* and *BAHD III* F4 alleles at these two positions. In addition, the close colocalization of QTLs with the position of the bm6 mutation should lead to consider the corresponding bm6 underlying determinant as also involved in the high cell wall digestibility of the F4 line.

The unusual S/G ratio in F4bm3 and F4 COMT-AS, as well as the lack of increased 5-hydroxy-guaiacyl units in F4 COMT-AS (not still investigated in F4bm3), is probably a consequence of the reduced expression of both *ZmCOMT* and *ZmF5H1* genes in the regular F4 line. However, it could also be related to the reduced ZmPAL expression, considering the possible organization of lignin synthesis enzymes in metabolons as it was highlighted in Brachypodium with distinct pathways to lignin from phenylalanine and tyrosine (Barros et al, 2016). Possible variations in the respective importance of these pathways could also be assumed for the F7106 line which has an S/G ratio close to the one of bm3 lines, with a low proportion of syringyl units. F7106 has higher lignin content than F4 and nearly the same cell wall digestibility, but lower releases of esterified *p*-coumarate and ferulate. F4 and F7106 could thus be complementary models for investigating the major underlying determinants of lignification and cell wall digestibility in maize and grasses.

If definite conclusions cannot be fully drawn, the unusual low lignification and high cell wall digestibility of the F4 line is very likely first related to atypical functioning of *ZmMYB* and *ZmNAC* transcription factors. Several tools are currently available for validating these hypotheses, including BAC sequencing and comparisons of corresponding areas, comparative gene expressions, gene editing based on the Crispr/Cas9 strategy, and search for transposon-tagging mutants in Mutator (Mu) collections. Search for mutants and investigations in the model grass Brachypodium could also be considered (*Bradi1g61397*, *Bradi5g16917*, *Bradi2g33980* are orthologs of *ZmMYB19*, *ZmSWN6*, and *ZmBAHD-III,* respectively). The identification of key-genes involved in cell wall digestibility variations will also facilitate marker assisted selection and the introgression of favorable alleles for silage maize (and other grass) improvement.

Facing an excess of milk production in the European Union as well as in other regions in the world, the reduction of production costs in dairy farming is an inescapable requirement for milk producers. Feeding costs could be considered as a privileged target for expense reduction, as new feeding approaches can be managed with shorter term strategies than costs corresponding to structural financial charges. In countries and seasons with favorable conditions for growth of meadow grasses, the use of grazing in dairy cow feeding can contribute to lowering feeding costs, a strategy which is nevertheless more difficult to manage with larger herds. Most often, maize silage indeed comprises the largest part of roughage in dairy cows diets. Increasing silage maize energy value is then a critical objective, allowing formulating diets with higher forage to concentrate ratios and reduced giving of costly energy concentrates. Moreover, hybrids with higher cell wall digestibility have increased and faster silage digestion in the digestive tract, with correlative higher silage intakes in cows. In addition, for more sustainable cropping conditions, a break in the search for continuous yield improvement should be considered, now focusing primarily maize breeding on feeding value and biotic and abiotic stress tolerances. Moreover, in numerous regions where climatic change induces higher temperature and lower rainfall, hybrids having a somehow reduced yield in comparison with currently available varieties, but with higher cell wall digestibility and energy value (and intake), will better fit with the near future water availability and cropping conditions. Feeding cows with silage maize hybrids of improved energy value will thus allow reducing both financial and environmental costs per animal or milk unit.

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