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QTL for floral stem lignin content and degradability in three recombinant inbred line (RIL) progenies of *Arabidopsis thaliana* and search for candidate genes involved in cell wall biosynthesis and degradability

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ABSTRACT

Deciphering the genetic determinants involved in cell wall assembly is a strategic issue for breeding programs that target both ruminant feeding and biofuel production. The *Arabidopsis thaliana* model system has great potentials to elucidate the genetic determinants involved in cell wall component biosynthesis and those involved in the regulation cascades allowing their coordinated assembly. QTL for biomass quality related traits (cell wall content, lignin content, and cell wall degradability) were mapped in the three *Arabidopsis* RIL progenies Bay0 × Shahdara, Bur0 × Col0, and Blh1 × Col0. Overall, 40 QTL were detected for these traits, explaining up to 33% and 12% of the observed phenotypic variation for lignin content and cell wall degradability respectively. Major QTL hotspots were mapped on chromosome 1 (position 5 Mbp), chromosome 4 (position 1 Mbp), and chromosome 5 (position 3 Mbp). A putative candidate gene set (82 genes) was considered including those previously described as involved in cell wall phenolic component biosynthesis, their regulation factors, and genes involved in lignified tissue patterning. Colocalisations observed (according to the reference sequence of Col0) between the detected QTL and these candidate genes did not prioritize any of the three gene groups (monolignol biosynthesis, transcription factors, lignified tissue patterning). Colocalizations were thus observed for 57% of monolignol biosynthesis related genes, 55% of the transcription factors considered, and 66% of genes considered to be involved in lignified tissue patterning and assembly. Colocalizations were observed for at least one member of all investigated gene families, except WRKY transcription factors. Colocalizations were also shown with several miRNA putatively involved in the regulation of lignifying tissue assembly. Taking into account the QTL shown in the Bur0 × Col0 progeny, allelic variations were shown in the MYB32, MYB58, MYB75, GRAS SCARECROW, AtC3H14 zinc finger, SHINE2, and IFL1 genes and in the AtMIR397a. Given that the list of candidate genes is not complete, and because the QTL support intervals encompassed genes of still unknown function, it is still not clear whether one of the selected candidates is responsible for the effect of a detected QTL. Mutant investigation and positional cloning steps are likely essential to clearly determine the causal mechanism involved in cell wall degradability variation.

Keywords: Arabidopsis; QTL; Lignin; Secondary Wall; Digestibility; Candidate Gene

1. INTRODUCTION

Lignocellulose biomass, which is the basis of herbivore nutrition, is also considered to be a major sustainable resource for second generation biofuel production. Lignocellulose biomass is made up of secondary walls and is mostly comprised of cellulose and hemicelluloses embedded in a phenolic component matrix. Lignification is the basic cause of reduced digestibility of forages in cattle digestive tracks [1,2] and of reduced fermentation of plant biomass into ethanol during green fuel production based on straw or stover materials [3-5]. In addition to lignin content, lignin structure and lignin associations with other cell wall components, including covalent linkages between phenolics and hemicelluloses, greatly in-

fluence cell wall properties [6,7]. Embedding between phenolics and carbohydrates thus prevent physical access of enzymes to cell wall carbohydrates and strongly limit their enzymatic hydrolysis. However, lignins and cross-linkages between cell wall components are essential as they contribute to the mechanical properties of tissues and impart hydrophobicity to vascular elements, allowing water and nutriment transportation.

Most forage and non-woody plants devoted to energy production for cattle feeding, biogas or biofuel production belong to the grass family. Nevertheless, the small mouse cress dicotyledonous *Arabidopsis thaliana* (abbreviated Arabidopsis) is a relevant model system to study the genetic basis of cell wall recalcitrance to saccharification. The monolignol biosynthesis pathway indeed has been extensively deciphered in Arabidopsis, with investigations most often based on mutant or deregulated plants. Another great interest of the Arabidopsis model system is related to the possible searches for regulation cascades of genes involved in cell wall component biosynthesis and genes controlling the cell wall assembly. Comprehensive investigations have thus described the transcription factor network regulating the organization of secondary cell wall biosynthesis in Arabidopsis [8,9].

The Arabidopsis lignified cell wall (type I cell wall as in all dicots and non grass monocots) is a composite material with phenolics, cellulose microfibrils, an amorphous matrix consisting predominantly of hemicelluloses (mainly xyloglucans and a few glucuronoxylans), and, to a lesser extent, pectins (rhamnogalacturonan I and II, and homogalacturonan). As in other dicotyledonous plants, Arabidopsis lignins are comprised of guaiacyl (G) units derived from coniferyl alcohol, syringyl (S) units derived from sinapyl alcohol, together with very low levels of *p*-hydroxyphenyl units (H) derived from *p*-coumaryl alcohol. The average relative frequencies of each monomeric units released by thioacidolysis of lignins of mature floral stems were shown ranging from 72% to 78% for G units and from 21% to 28% for S units, with less than 1% of H units [10-12]. Interfascicular fibers have a higher content in S units than vascular bundles [11]. Incorporation of ferulic acid in lignins has also been shown to occur in Arabidopsis, but in very low amount, nearly 100 times lower compared to maize [13,14]. Conversely to grasses, there is a quasi-lack of *p*-coumaric acid in Arabidopsis cell wall. Moreover, grass hemicelluloses mostly comprised glucurono-arabinoxylans, with very little pectins. Given the differences between type I and type II cell walls, it should be considered that different gene families will likely only be found in grass genomes, while several ortholog genes will have different functions in grass and dicot plants. According to Carpita and McCann [15] and to Bosch *et al.* [16], nearly two-third of cell wall related genes should be common in grasses and Arabidopsis.

A more complete understanding of the molecular networks involved in cell wall assembly is essential for further efficient breeding of plants with lignocellulosic biomass that can be more easily broken-down in animal digestive tracks or industrial bio-fermenters. QTL for cell wall related traits have been mostly detected in maize (reviews by Barrière *et al.* [6,17]) and to a lesser extent in woody species such as pine [18-20], poplar [21], and eucalyptus [22-24]. No studies have yet validated genes as responsible for the effect of the QTL, even if colocalizations between candidate genes and QTL were investigated in maize, poplar, and eucalyptus [17,24-26]. While many investigations related to cell wall assembly and cell wall component biosynthesis have been done in Arabidopsis based on mutant studies, no QTL analyses have been considered on this topic despite the early availability of the genomic reference sequence for this species, apart from two in the Bay0 × Shahdara progeny. One was devoted to primary wall carbohydrates [27] and one to floral stem lignification [28]. The objective of this work was thus to detect QTL involved in floral stem lignification in three Arabidopsis RIL progenies with a systematic search for candidate genes underlying each QTL. The list of candidate genes considered mainly focused on a limited set of genes which had been described with key-roles in constitutive phenolic component biosynthesis, in the regulation of phenolic component biosynthesis, or in the assembly of lignified tissues.

2. MATERIAL AND METHODS

2.1. RIL Progeny Description

The three recombinant inbred lines were derived at INRA Versailles by single seed descent (SSD) up until the F7 generation from crosses between Bay0 and Shahdara, Col0 and Bur0, and Blh1 and Col0 ([29], and VNAT database, http://dbsgap.versailles.inra.fr/vnat/). According to data available in the VNAT database, the Bay0 ecotype originates from a fallow-land habitat near Bayreuth (Germany) and the Shahdara ecotype was collected in the Pamiro-Alay mountains of Tadjikistan in the drainage basin of the Shakdara river. The Bur0 ecotype was collected from the roadside nearby Burren (Eire) and the sequenced ecotype Columbia descended from Landsberg which originates from Gorzow Wielkopolski (Landsberg/Warthe, Poland). Blh1 is an ecotype from Bulhary (Czechoslovakia) that was collected in a grassy place on the left riverside of the Thaya. The diversity in geographical and ecological origins of ecotypes is such that one would expect a significant allelic diversity that is especially well adapted to QTL investigations.

The genetic map of the Bay0 × Shahdara RIL progeny

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was originally based on 38 microsatellite markers [30], but it was further extended to 69 markers on the 420 RIL. The average genetic distance between two adjacent markers was thus 6.1 cM, corresponding on average to 2.0 Mb, with a global good allelic equilibrium (Bay0 51%, Shahdara 49%, VNAT database). This progeny was the basis of numerous QTL analyses including a first study of cell wall related QTL, which was based on the 38-marker map with a less elaborated NIRS calibration of cell wall traits [28]. The Bur0 × Col0 RIL progeny comprised 343 RIL genotyped with 87 markers. The average distance between markers was 4.4 cM, corresponding on average to 1.4 Mb, with a global good allelic equilibrium (Col0 51.3%, Bur0 48.7%, VNAT data- base). The Blh1 × Col0 progeny comprised 315 RIL successfully genotyped with 75 markers. The average distance between markers was 5.9 cM, on average equivalent to 1.65 Mb, with a convenient global allelic equilibrium (Col0 52.7%, Blh1 47.3%, VNAT database).

2.2. Plant Cropping

Plants were grown in a greenhouse at INRA Versailles (France), in a mix of sand and compost, and watered with a normal nutrient solution (harvested in 2001 for Bay0 × Shahdara, in 2005 for Bur0 × Col0 and Blh1 × Col0). The temperature was 23˚C during the day and 15˚C at night, with a 16 h light/8 h dark photoperiod. Additional light was added according to natural day length giving 105 μE/m^2/s at the plant level. Floral stems were collected at seed maturity in successive harvests according to plant earliness. Only one replicate was cropped, but growth conditions ensured homogeneous materials allowing relevant phenotypic analysis.

2.3. Samples Analysis and Trait Variation

Floral stem samples cleared of siliques were dried in a ventilated oven (65˚C). Dry samples were then ground with a hammer mill to pass through a 1 mm screen for later analyses. Due to sample quantity available after grinding, investigations were carried out on 396 RIL out of 420 in Bay0 × Shahdara, 311 out of 344 in Bur0 × Col0, and 299 out of 315 in Blh1 × Col0. Plant cell wall estimated constituents included neutral detergent fiber (NDF, [31]) which is an estimate of cell wall content, Klason lignins (KL, [32]) which is an estimate of the whole lignins, acid detergent lignins (ADL, [31]) which is an estimate of the core acido-resistant fraction of lignins [33], and neutral detergent soluble fiber (NDSF, [34]) which is an estimate of pectins. ADL and KL, which are constituents of the cell wall, were both expressed as percentage of NDF (ADL/NDF and KL/NDF). The *in vitro* dry matter degradability (IVDMD) was estimated based on enzymatic solubility of samples according to Aufrère and Michalet-Doreau [35]. Cell wall degradability was first considered according to Struik [36] and Dolstra and Medema [37] as the *in vitro* NDF degradability (IVNDFD), which is computed assuming that the non-NDF part of plant material is completely digestible [IVNDFD = 100 × (IVDMD – (100 – NDF))/NDF]. Cell wall degradability was also estimated according to the DINAGZ trait which has been proposed and used for maize plants by Argillier *et al.* [38] and Barrière *et al.* [39], considering that starch (St), soluble carbohydrates (SC), and crude protein (CP) are fully digestible [DINAGZ = 100 × (IVDMD – St – SC – CP)/(100 – St – SC – CP)]. Because no starch is present in Arabidopsis mature stems and because conversely to maize significant amounts of soluble pectins are present, starch content was replaced in the DINAGZ formula by NDSF content. For DINAGZ estimate, crude protein was estimated according to Kjeldahl nitrogen × 6.25, and soluble carbohydrates according to Lila [40]. All plant components and IVDMD were estimated using near infrared reflectance spectroscopy (NIRS, NIRS system 6500 spectrophotometer, FOSS technology). Calibration equations were developed at INRA Lusignan (**Table 1**), and calibration regressions were validated with laboratory analysis of nearly 40 samples per progeny.

Table 1. Characteristics of NIRS calibrations developed for cell wall traits in Arabidopsis mature stems (for each trait, number is the numbers of analyzed samples with their mean and standard deviation (St Dev), RSQ is the coefficient of determination between laboratory analysis and NIRS prediction, and SECV is the standard error of cross validation prediction; DM = dry-matter).

	number	Mean	St Dev	RSQ	SECV
IVDMD (in vitro DM degradability)	749	54.9	12.1	0.99	1.50
NDF (neutral detergent fiber)	732	52.5	10.7	0.98	1.80
ADL (acid detergent lignin)	272	8.7	1.7	0.94	0.46
KL (Klason lignin)	733	12.2	2.7	0.88	1.00
Crude protein	531	11.4	7.6	0.99	0.94
Soluble carbohydrates	246	1.8	1.6	0.60	1.04
NDSF (neutral detergent soluble fiber)	115	11.4	2.2	0.70	1.36

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Lower numbers of reference analyses were available for ADL, NDSF, and SC. However, fairly low coefficients of determination between laboratory analysis and NIRS prediction were only observed for NDSF and SC, two traits that were only involved in the estimate of the DINAGZ cell wall trait. Moreover, the average content in SC was very low in plant mature stems. Pearson's phenotypic correlations between traits were computed for each progeny.

2.4. QTL Mapping and Candidate Gene Investigations

QTL mapping was based on individual values of each investigated RIL in each progeny, using the Composite Interval Mapping method (CIM) implemented in the PLABQTL computer package [41]. PLABQTL uses the regression method of Haley and Knott [42] in combination with selected markers as cofactors. Cofactors are selected by stepwise regression (option cov SELECT) with an "F-to-enter" and an "F-to-delete" value of 7, retaining markers significant at the 1% level. Lod thresholds are obtained by the permutation test method (1000 permutations, [43-45]). Percentages of phenoltypic variance ascribed to individual QTL are estimated with the approximate standard error of Kendall and Stuart [46]. LOD support intervals are constructed for each QTL according to Lander and Botstein [47], which could be underestimated in the case of CIM. The additive ef- fects of QTL are estimated as half the difference between the phenotypic values of the respective homozygotes. Physical QTL positions (Mbp, Mega-base-pairs) were estimated based on physical positions of the two flanking markers assuming a constant and linear relationship between recombination and physical distances within this interval. Physical lengths of QTL support intervals were similarly estimated. A consensus map was drawn with MapChart, based on physical distances (http://www.biometris.wur.nl/uk/Software/MapChart/).

The list of putative candidate genes was established gathering available information on genes involved or putatively involved in the lignified cell wall assembly of Arabidopsis. This list thus comprised Arabidopsis genes belonging to the monolignol pathway, their transcription factors, and genes involved in lignified tissue assembly. The list of putative candidate genes also comprised orthologs of genes for which an involvement in cell wall lignification and deposition have been established in other dicotyledonous plants including poplar [48] and eucalyptus [49], but also maize [6]. The list took into account Arabidopsis expression data (http://genecat.mpg.de/; http://atted.jp, [50-53]) and only genes with expression in stems were considered. Gene physical positions were those given in "The Arabidopsis Information Resource" database (TAIR, http://www.arabidopsis.org).

2.5. Allelic Variation Investigations

Alleles of the Bur0 and Col0 genomes were compared according to sequence data available in the public databases, with several validation based on direct sequencing. The sequence of the reference Col0 ecotype is available in the TAIR database (www.arabidopsis.org/) and the sequence of the Bur0 ecotype has been recently released [54,55] in the 1001genomes database (www.1001genomes.org/).

3. RESULTS AND DISCUSSION

3.1. Arabidopsis Genes Considered as Putative Candidates Underlying Cell Wall Trait QTL

3.1.1. Genes Involved in Monolignol Biosynthesis and Polymerization

The monolignol pathway has been extensively investigated in Arabidopsis, making it easy to list the genes involved [10,13,56-63]. After deamination of phenylalanine by phenylalanine ammonia lyases (PAL), monolignol biosynthesis is the result of successive steps of hydroxylation and methylation on the aromatic ring. Most of genes in the pathway belong to small multigene families, with different members likely involved in different metabolons. As far as information was available, the considered candidate gene list included only genes involved in constitutive lignification and excluded genes involved in lignification after non developmental signals such as abiotic or abiotic stresses (**Table 2**).

Class III peroxidases and laccases, which are both involved in monolignol polymerization, belong to larger multigene families. The considered members were chosen based on their expression in stem and/or lignified tissues [12,64-71]. There is an important redundancy of peroxidase genes and most often individual mutants did not clearly exhibit any lignin modified phenotype. Nevertheless, redundancy importance is likely reduced by the fact that many peroxidases have tissue specific expression patterns [72].

Before their polymerization, monolignols are transported from the cytosol to the cell wall, likely as monolignol glucosides [73,74]. Two uridine-diphosphate-glucosyltransferases (UGT) have been shown with capacity to glucosylate coniferyl and sinapyl alcohols [75, 76]. The release of monolignol aglycone from its glucosidic form at the cell wall for subsequent lignin polymerization is thought to be mediated by specific glucosedases. Arabidopsis β-Glu45 and β-Glu46 β-glucosidases (UGT), which are strongly expressed in lignifying organs, encode proteins with narrow specificity towards the three monolignol glucosides [74]. In addition, and even if this

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Table 2. List of considered candidate gene underlying cell wall related QTL. Genes involved in monolignol biosynthesis and polymerization.

Gene name and function	TAIR Arabidopsis gene number
PAL	At2g37040, At3g10340, At3g53260
4CL	At1g51680, At3g21230, At3g21240
C4H	At2g30490
C3'H	At2g40890
HCT	At5g48930
CCoAOMT	At4g34050
CCR	At1g15950
COMT	At5g54160
F5H	At4g36220
CAD	At3g19450, At4g34230
UDP glucosyltransferase (UGT)	At5g26310, At5g66690
β-glucosidase	At1g61810, At1g61820
ABC transporter	At1g59870, At2g37280, At3g16340, At3g25620
Peroxidase	At3g49120, At4g21960, At4g37530, At5g06720, At5g42180, At5g51890
Laccase	At2g29130, At2g38080, At5g01190, At5g03260, At5g05390, At5g60020

Table 3. List of considered candidate gene underlying cell wall related QTL. Transcription factors involved in secondary wall biosynthesis and assembly.

Gene name and function	TAIR Arabidopsis gene number
MYB	At1g16490, At1g22640, At1g56650, At1g79180, At3g08500, At4g01680, At4g22680, At4g34990, At4g38620, At5g12870
NAC	At1g28470, At1g32770, At1g71930, At2g46770, At3g61910, At4g28500, At5g09330, At5g13180, At5g62380, At5g64530
bHLH	At1g61660, At2g43060, At3g07340, At5g48560
WRKY	At2g30590, At2g44745, At4g31550, At4g39410
KNAT	At1g62990, At1g70510, At4g08150
LIM zinc finger	At1g10200, At3g55770
CCCH, C3HC4 zinc finger	At1g06040, At1g66810, At1g68200, At1g72200, At1g72220, At2g20650, At3g10910, At3g47990, At3g60080, At5g42820
GATA, HD zinc finger	At1g74660, At5g25830
bZIP	At4g35040, At5g24800
LBD/ASL protein	At1g07900, At1g31320, At2g28500, At2g40470, At2g45420, At3g49940, At4g00220

step is not fully elucidated, ABC transporters are very likely involved in the transport of monolignols across membranes [66,77-80].

3.1.2. Transcription Factors Involved in Regulation of Lignified Cell Wall Biosynthesis Genes

Comprehensive investigations have used the Arabidopsis model system in order to document the transcription factors regulating the secondary wall biosynthetic program (**Table 3**).

Different Arabidopsis MYB and NAC proteins regulate the expression of several transcription factors and/or genes involved in secondary cell wall biosynthesis [8,9, 81-85]. NAC NST1 and NST2 (secondary wall thickening promoting factor), SND1 (secondary wall associated NAC domain protein 1), and VND6 and VND7 (vascular-related NAC domain) are thus cell-type specific and partly functionally redundant "master" genes activating the entire secondary wall biosynthetic pathways [81,82]. In addition, VNI2 (VND-INTERACTING2, and to a lower extent VNI1), is a transcriptional repressor of vessel-specific genes regulated by VND7 [86]. Interactions mostly occur between VNI2 and VND7, but also exist to a lower extent with other VND proteins and possibly other NAC factors. Downstream these genes, direct target transcription factors such as AtMYB46 and AtMYB83, and several secondary targets regulating monolignol biosynthesis genes were also considered to be relevant candidates [9,84,86-90].

Complementarily, different LIM and WRKY transcription factors have to be considered as candidate genes. The tobacco NtLIM1 gene was shown to be a positive regulator of the lignin pathway [91]. LIM proteins are characterized by zinc-binding domains that ligate two zinc ions. Unlike the classical zinc fingers, these domains do not bind DNA, but mediate interactions with other proteins [92]. WRKY proteins are involved in many processes, especially including defense against biotic stresses and their role in cell wall constitutive lignification is not yet established [93,94]. However, several AtWRKY genes were highly expressed in lignifying stems, and the mutation of the AtWRKY12 gene induced secondary wall formation of pith cell [53]. Several WRKY genes expressed in healthy lignifying stems were thus considered as candidates.

Transcription factors belonging to other families have been related to lignified tissue biosynthesis, and could also consequently be considered as candidates underlying lignified cell wall related QTL. Zinc finger proteins, which constitute one of the largest families of transcriptiontion factor regulatory proteins, are involved in numerous regulations of plant development, including lignified

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tissue deposition. Zinc-finger C2H2 genes were the most frequently represented transcription factors in eucalyptus secondary xylem libraries [49]. The CCCH zinc finger protein (AtC3H14) has been shown to activate all of the secondary wall phenolics and carbohydrate related genes tested [95]. Both SND1 and MYB46 proteins bound to the AtC3H14 promoter, and AtC3H14 may function as master regulator of secondary wall biosynthesis, located downstream of MYB46.

Basic region/leucine zipper motif (bZIP) transcription factors regulate several processes during plant development and are involved in biotic and abiotic stress responses. More especially, studies of group I bZIP genes have given converging evidence that members of this family might regulate vascular development [96].

LBD or ASL (LATERAL ORGAN BOUNDARIES DOMAIN or ASYMMETRIC LEAVES) proteins are a family of plant-specific transcription factors with 43 members in Arabidopsis which regulate a variety of developmental processes [97]. ASL19/LBD30 and ASL20/LBD18 are expressed under the control of VND6 and VND7, and LBD18 or LBD30 over-expression induced trans-differentiation of cells from non-vascular tissues into tracheary-like cells [98]. These LBD proteins appear to be involved in a feedback loop with VND7 that regulates genes involved in tracheary element differentiation [98,99]. In addition, Yordanov *et al.* [100] showed similar results in poplar based on investigations in a LBD1 mutant which displayed an increased secondary phloem. The LBD1 and LBD11 Arabidopsis genes are the closest orthologs of poplar PtaLBD1.

Basic helix-loop-helix (bHLH) transcription factors belong to a family of transcriptional regulators for which many different functions have first been identified in animals, including the control of cell proliferation and development of specific cell lineages. Their mechanism for controlling gene transcription often involves homodimerization or heterodimerization. At least 133 bHLH genes have been shown in Arabidopsis and the AtbHLH gene family also constitutes one of the largest families of transcription factors in this species [101]. Genes considered in the candidate gene list were those co-expressed with lignin related genes and significantly expressed in stems.

3.1.3. Candidate Genes Putatively Involved in Lignified Tissue Patterning

The COV1 (continuous vascular ring) gene encodes an integral membrane protein of unknown function which is supposed to be involved in a mechanism that negatively regulates the differentiation of stem vascular tissue by a mechanism independent of auxin [102]. The Arabidopsis HCA (HIGH CAMBIAL ACTIVITY) mutant also exhibits an increase in vascular tissue development characterized by a continuous ring of xylem and phloem with an unusual cambial activity. Conversely to COV1, HCA was shown to control the arrangement of vascular bundles by regulating the auxin-cytokinin sensitivity of vascular cambial cells [103]. Another mutant with high cambial activity (HCA2) has been described [104]. Its phenotype was shown to result from an elevated expression of a DOF transcription factor (DOF5.6, DNA binding with one finger), preferentially expressed in the cambium, phloem, and interfascicular parenchyma cells of inflorescence stems. Ectopic lignification was also related to variation in DOF gene expression in pom1, eli1 (ectopic lignification 1) and det3 (de-etiolated 3) Arabidopsis mutants, in addition to expression variation of MYB genes [105] (**Table 4**).

The GRAS SCARECROW and SCARECROW-like proteins belong to a plant specific transcription factor family which contains basic leucine zipper regions [106, 107]. These proteins are involved in complex regulatory pathways regulating tissue patterning and differentiation [107]. The SCARECROW protein is thus 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 [108].

Members of a small class III homeodomain-leucine

Table 4. List of considered candidate gene underlying cell wall related QTL. Genes involved in lignified tissue patterning.

Gene name and function	TAIR Arabidopsis gene number
COV	At1g43130, At2g18460, At2g20120
HCA	At4g(unknown)
GRAS Scarecrow-like and SHR	At1g21450, At1g50420, At1g50600, At3g54220, At4g17230, At4g37650
DOF-type zinc finger	At1g21340, At1g64620, At1g67030, At2g28200, At3g21270, At3g47500, At5g60850, At5g62940
Homeodomain-leucine zipper	At1g30490, At1g52150, At2g34710, At4g32880, At4g37790, At5g60690
Kanadi	At5g16560
ERF/AP2 subfamily B6 SHINE	At1g15360, At5g11190, At5g25390
Shatterproof	At3g58780
ROP/RAC/RAS/RAB GTPase	At1g08340, At1g22740, At1g73640, At3g18820, At4g39990, At5g03520, At5g45970, At5g62880
Xylem Cysteine Protease	At1g20850, At4g35350
Xylem Serine endoPeptidase	At4g00230
Bcl2-associated athanogene	At3g51780, At5g07220, At5g52060
Microtubule Associated Protein	At1g68060, At4g17220

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zipper family out of which AtHB8, AtHB9 (PHAVOLUTA), AtHB14 (PHABULOSA), AtHB15 (CORONA), and IFL1 (REVOLUTA), are expressed in vascular tissues and have been shown to play regulatory roles in vascular differentiation [109-114]. The IFL1 Arabidopsis gene has two maize orthologs of which mutants have rolled leaf phenotypes (RLD1 and RLD2). The maize RLD1 gene is regulated by the ZmmiR166 miRNA [115]. The expression of the aspen PtaHB1 gene, which is also orthologous to IFL1, is also inversely correlated with the level of miR166 miRNA [116]. Similarly, the AtmiR166g over-expression in Arabidopsis jabba-1D (jba-1D) mutant plants affects the transcripts of several class III AtHD-ZIP family target genes. PHABULOSA, PHAVOLUTA, and CORONA genes have thus a significantly reduced expression in the jba-1D background, while REVOLUTA expression is increased and ATHB8 expression is unchanged [117]. In addition, interactions between HDZIP III and KANADI gene family members were shown to be involved in the establishment of the spatial arrangement of phloem, cambium and xylem. It was considered that HDZIP III and KANADI transcripttion factors control cambium activity, with KANADI proteins acting on auxin transport, and HDZIP III proteins promoting axial cell elongation and xylem differentiation [114]. These genes could therefore be considered as candidates even if their involvement occurs upstream in the pathway.

The SHP1 (SHATTERPROOF MADS-box) gene, which has been shown to specify with SHP2 the lignified valve margin of mature Arabidopsis siliques [118], likely has other roles in tissue lignification as it is also expressed in stems and down-regulated at the maturing stage [51]. Moreover, an ortholog exist in maize (ZmZAG5), the function of which is not known [6,119,120].

Two microtubule-associated proteins (AtMAP70-5 and AtMAP70) were shown to be essential for defining where secondary cell wall polymers are applied at the cell cortex in wood-forming cells [121]. These two genes were consequently added to the candidate gene list.

A member of the plant ROP family (EgROP1) was shown to be preferentially expressed in the cambial zone and differentiating xylem of eucalyptus. Its over-expression in Arabidopsis altered vessel formation and fibre growth in secondary xylem [122,123]. AtROP/AtRAC/AtRAB genes encode geranyl-geranylated GTP-binding proteins (GTPases) involved in the auxin proteolysis pathway. The latter are thought to provide a universal mechanism in the control of extracellular signal transmission to intracellular metabolic pathways related to growth, differentiation, development and defense responses [124,125]. Several of them are involved in autophagy and xylem development [126]. Eight Arabidopsis RAS/RAC/RAB GTPases expressed in stems were thus considered as candidate genes.

Candidate genes were also considered in the ERF/AP2 (ethylene responsive factor/APETALA2) SHINE1 family. Based on over-expression investigations in rice [127], an ERF/AP2 gene was indeed considered as an upstream transcriptional regulator of both master and secondary target genes involved in the biosynthesis of cell wall phenolic and carbohydrate components. It was suggested that this ERF/AP2 transcription factor directly binds to promoter regions of NAC and MYB genes involved in regulation of cell wall assembly. Rice plants over-expressing Arabidopsis SHINE2 gene had lower lignin and higher cellulose and hemicellulose contents, without changes in plant strength and overall performances.

Finally, candidate genes were considered in families involved in proteolysis and apoptosis, which are key-steps in the lignification process. Plant BAG (Bcl-2-associated athanogene) proteins are multi-functional and they regulate apoptotic-like processes in plant development and responses to pathogen attack or abiotic stress [128]. Two cysteine protease genes (XCP1, XCP2), highly up-regulated in maturing stems, and one xylem serine peptidase (XSP1) were considered as putative candidates underlying cell wall QTL [51,88,129].

3.1.4. miRNA Involved in Regulation of Cell Wall Related Genes as Candidates Underlying QTL

Several AtMIR were considered as possible candidate following the description of their involvement in lignin-related gene regulation (**Table 5**). AtMIR165 and AtMIR166 have been shown to target and regulate the transcription class III AtHD-ZIP genes, including those involved in vascular tissue differentiation [115,130-133]. AtMIR397a and b, AMIR408, and AtMIR857 control laccase genes, some of them potentially involved in monolignol polymerization in the cell wall through the regulation of copper homeostasis [134]. In addition, AtMIR858 targets the MYB83 gene which interacts with VND7 and negatively regulates xylem vessel formation [132].

Table 5. List of considered candidate gene underlying cell wall related QTL. miRNA regulating lignified tissue biosynthesis and assembly.

AtMIR	TAIR Arabidopsis gene number
AtMIR165	At1g01183, At4g00885
AtMIR166	At2g46685, At3g61897, At5g08712, At5g08717, At5g41905, At5g43603, At5g63715
AtMIR397	At4g05105, At4g13555
AtMIR408	At2g47015
AtMIR857	At4g13554
AtMIR858	At1g71002

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3.1.5. The Final Candidate Gene List

Ultimately, 133 putative candidate genes were considered (**Table 2**), out of which 39, 18, 22, 23, and 31 were located on chromosomes 1, 2, 3, 4, and 5, respectively, while 12, 5, 1, 11, and 11 QTL were located on the same chromosomes, respectively. Out of the 16 putative genes involved in constitutive monolignol biosynthesis, 2, 3, 5, 4, and 2 are located on chromosomes 1, 2, 3, 4, and 5, respectively without any clustering in the monolignol pathway or between members of multigene families. Six peroxidase genes out of the 73 described in the Arabidopsis genome [64], and six laccase genes out of the 17 described in the Arabidopsis genome [65], were considered. These were mostly located on chromosomes 4 and 5. Transcription factors considered as putatively involved in regulation of cell wall phenolic component biosynthesis were mostly located on chromosome 1 (19 out of 53), while the genes involved in lignified tissue patterning were mostly located on chromosomes 1 and 5 (15 and 11 out of 44, respectively). This candidate gene list, which focused on genes involved in cell wall phenolic component biosynthesis and regulation, cannot be considered as exhaustive. Several other genes, whose roles in cell wall lignification and deposition have not yet been defined, should be added to the list of candidates underlying cell trait related QTL. Moreover, a few genes encoding proteins of unknown function, which were expressed in stems, colocalized with QTL. However, the latter were not taken into account as their possible involvement in lignified tissue biosynthesis and deposition is still unknown.

3.2. Trait Phenotypic Variation and Correlations in RIL Progenies

Observed means and ranges of variation were nearly similar for cell wall traits in the three RIL progenies (**Table 6**), with nevertheless some distinctive characteristics between families. NDF content was lower in the Bay0 × Shahdara progeny. Average ADL lignin content was nearly 3 percent higher in Bur0 × Col0, with correlatively lower cell wall degradabilities. Unlike with ADL lignins, no differences between progenies were observed for average Klason lignin content. Lignin polymers in the different progenies were thus characterized by the loss of a variable acid-soluble part during the first step of the ADL procedure [33,135].

Correlations were high between cell wall content and other traits, except with Klason lignins (**Table 7**, correlations significantly different from zero at P = 0.01 for absolute values higher than 0.15). These results highlighted an important difference between Arabidopsis and maize. In Arabidopsis cell wall degradability was highly related to cell wall content, with an average correlation value equal to –0.85, while it was only 0.13 in maize, as an average of two RIL progenies in *per se* value and topcross experiments ([17,136] and unpublished data). However, the cell wall degradability was similarly related to ADL lignin content in maize (average r = –0.80) and Arabidopsis (average r = –0.70). The weak or null relationship between Klason lignin and cell wall degradability in both maize and Arabidopsis strengthened the role of only the core lignin part (estimated as ADL/NDF) in the inhibition of cell wall degradability. The lack of a secondary cambium and the different tissue organization between grasses and dicotyledonous plants partly limit gene discovery when using Arabidopsis as a model system to investigate crop plants such as maize. However, major determinants of lignification and correlative negative effects on cell wall degradability can likely be considered in Arabidopsis to identify candidate genes in maize.

3.3. QTL Analyses in the Three RIL Progenies

Lod thresholds equal to 2.3 and 3.0, 2.5 and 3.3, and 2.5 and 3.5 yielded experiment-wise error rates equal to 5% and 1% for Bay0 × Shahdara, Bur0 × Col0, and Blh1 × Col0, respectively. QTL were therefore only considered significant in each progeny for error equal to or lower than 5%, and overall 40 QTL were mapped (**Figure 1**). Because data were available from only one replicate per RIL, the total percentages of variance explained by all QTL for each trait in each progeny were moderate and

Table 6. Means, standard deviations, and extreme values for investigated traits in the three RIL progenies.

Trait	Bay0 × Sha (396 RIL)				Bur0 × Col0 (311 RIL)				Blh1 × Col0 (299 RIL)			
	Mean	St Dev	mini	maxi	Mean	St Dev	mini	maxi	Mean	St Dev	mini	maxi
NDF	47.3	6.1	31.3	58.3	60.6	3.7	43.3	68.2	58.5	3.9	47.9	68.9
ADL/NDF	14.0	1.4	9.3	17.7	17.2	0.8	12.9	19.5	14.7	1.1	11.9	18.8
KL/NDF	24.4	2.0	20.4	32.2	23.1	1.3	19.3	26.6	23.7	1.4	19.3	26.8
DINAGZ	44.4	5.9	32.4	68.6	37.9	4.1	28.1	60.8	41.4	4.3	30.8	53.7
IVNDFD	20.3	5.1	9.6	41.7	15.3	3.5	7.9	39.3	22.8	3.7	13.1	34.1

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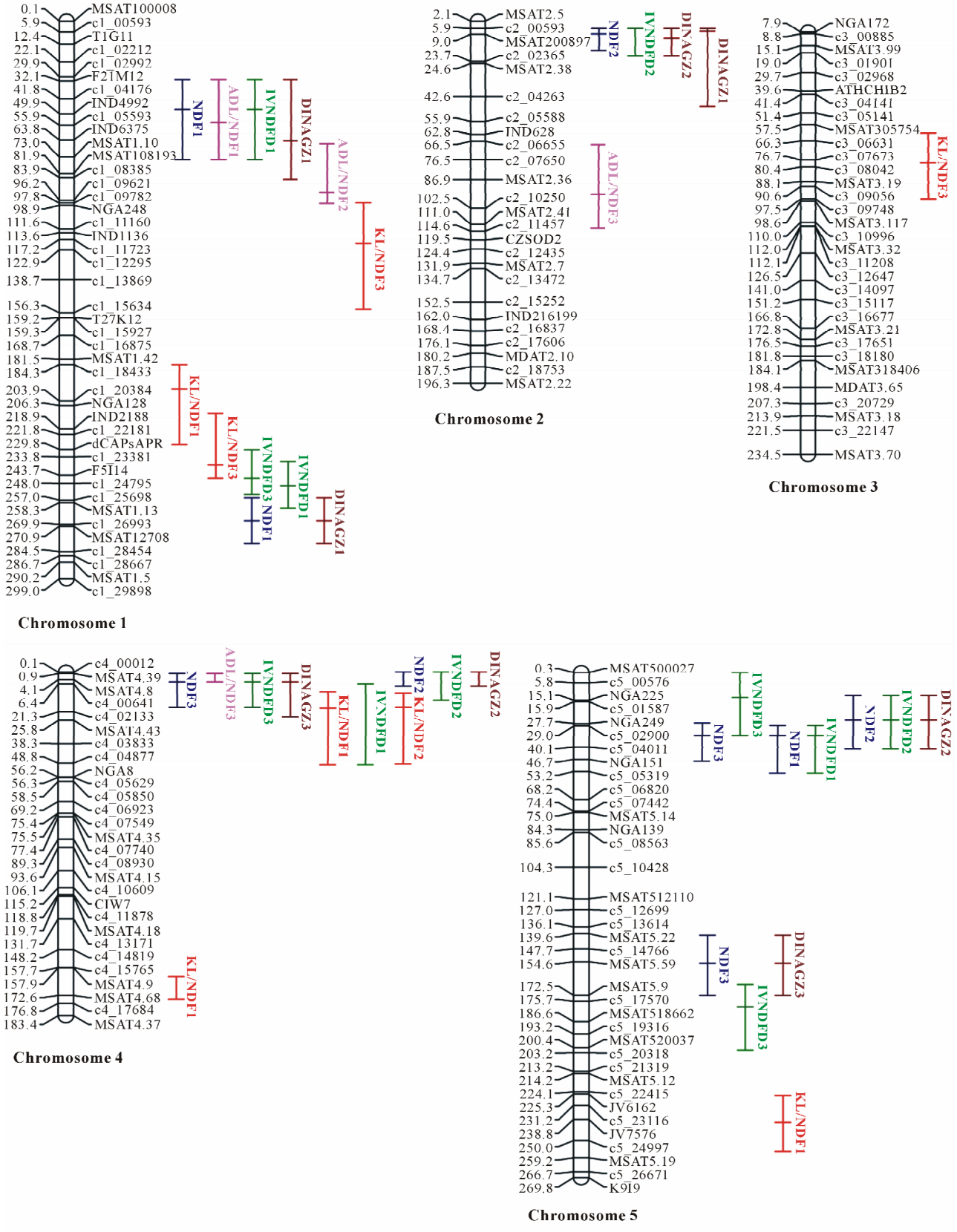

Figure 1. Consensus map of the three RIL progenies based on physical distances (distances were given as 10^1 Mbp; numbers after QTL trait names indicated the respective progenies with 1 = Bur0 × Col0, 2 = Blh1 × Col0, 3 = Bay0 × Shahdara).

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Table 7. Correlations between investigated traits in the three RIL progenies.

Trait	NDF	ADL/NDF	KL/NDF	DINAGZ
Bay0 × Sha				
ADL/NDF	0.70			
KL/NDF	–0.29	0.17		
DINAGZ	–0.84	–0.54	0.24	
IVNDFD	–0.78	–0.63	–0.07	0.75
Bur0 × Col0				
ADL/NDF	0.72			
KL/NDF	0.02	0.38		
DINAGZ	–0.89	–0.76	–0.01	
IVNDFD	–0.89	–0.80	–0.07	0.92
Blh1 × Col0				
ADL/NDF	0.63			
KL/NDF	0.01	0.23		
DINAGZ	–0.92	–0.67	0.08	
IVNDFD	–0.79	–0.77	0.08	0.90

nearly equal to 20%, 30%, and 30% in the Bay0 × Shahdara, Bur0 × Col0, and Blh1 × Col0 progenies, respectively. The lack of experimental replicates thus induces some inaccuracies in the phenotypic values of RIL with consequently a lowering of QTL Lod values and lack of detection of QTL of weaker effects. Eight QTL explaining more or at least 10% of the observed phenoltypic variation were nevertheless shown, with corresponding Lod values equal or greater than 6.0. In addition, no significant interactions between any QTL were observed.

Only 5 QTL out of 40 were mapped in isolated position, including one ADL/NDF QTL in the Bay0 × Shahdara progeny, and four KL/NDF QTL in Bur0 × Col0 and Bay0 × Shahdara progenies (**Table 8**). Half of KL/NDF QTL was thus located in isolated positions, strengthening the relative independence between Klason lignins, ADL lignins or cell wall degradability. Two locations on chromosomes 4 and 5 brought together 15 QTL for the different cell wall related traits observed in the three RIL progenies. Four locations including three on chromosome 1 and one on chromosome 2 brought together 15 QTL for the different traits and two RIL progenies, while three QTL were only observed for the Bay0 × Shahdara progeny on chromosome 5.

Most QTL with the highest Lod values were observed in the Bur0 × Col0 progeny, probably reflecting larger differences in cell traits between these two ecotypes. However, a reverse situation was shown on the upstream part of chromosome 4, with QTL of high Lod values for Bay0 × Shahdara and Blh1 × Col0 RIL, and lower values for Bur0 × Col0 RIL. In addition, a Klason lignin QTL with a Lod value equal to 26.4 and explaining 33.4% of the phenotypic variation for this trait was shown at position 1.9 Mbp in the Blh1 × Col0 progeny. The existence of two genetic traits underlying QTL in positions 0.3 and 1.9 Mbp was strengthened by the fact that alleles increasing Klason lignin content and cell wall degradability originated from the same parent (Col0, based on additive values) in the Blh1 × Col0 progeny.

Plant resistance to pests and diseases included both specific and general mechanisms, out of which increased lignin contents and tissue stiffness. Hence, an effort was made to identify relationships between cell wall QTL shown in the three investigated progenies and QTL previously shown and involved in pathogen resistance. On chromosome 5 in position 50 cM, a QTL of resistance to *Botrytis cinerea* was shown in the Bay0 × Shahdara progeny [137]. It colocalized with a QTL of cell wall degradability with Bay0 alleles increasing both disease susceptibility and cell wall degradability. In the Bur0 × Col0 progeny, a QTL controlling partial resistance to clubroot (*Plasmodiophora brassicae*) was shown on chromosome 5 in position 11 cM [138]. It similarly colocalized with a QTL of cell wall degradability with Col0 alleles increasing both disease susceptibility and cell wall degradability. Another QTL was shown on chromosome 1 in position 12 cM which colocalized with QTL of ADL lignin and cell wall degradability, the Col0 allele increasing disease susceptibility and cell wall degradability and the Bur0 allele increasing lignin content. Whether these colocalizations correspond to the same genetic determinant or to different genes in close positions is still unknown. However, a genetic mechanism inducing simultaneously greater cell wall degradability and greater disease susceptibility is a plausible hypothesis. The MYB58 and SHINE2 genes could be involved in these capacities

3.4. Colocalisation between QTL in the Three RIL Progenies and Putative Candidate Genes

3.4.1. Chromosome 1, QTL Mapped at 5.5 Mbp

Several highly relevant candidate genes colocalized with QTL of large effects detected at 5.0 Mbp on chromosome 1 (**Table 9**). AtCCR1 is an obvious candidate, owing to its important role in the lignin pathway as shown in studies of mutants or transgenic approaches in Arabidopsis [13,56] and in other species including tobacco [139], eucalyptus [140], poplar [14,141], spruce [142], and maize [143]. The second candidate to be considered

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Table 8. Putative QTL observed for lignin contents and degradability traits the three RIL progenies. Additive value of traits is positive when allele increasing trait value originated from Shahdara, Bur0, and Blh1, and negative when allele increasing trait value originated from Bay0, and Col0.

RIL progeny	Trait	chr - pos	pos Mbp	supp int cM	supp int Mbp	LOD	R2	add
Bur0 × Col0	NDF	1 - 10	4.7633	8 - 16	3.1389 - 7.4543	7.43	10.4	1.18
Bur0 × Col0	ADL/NDF	1 - 12	5.4546	8 - 16	3.1389 - 7.4543	16.18	21.3	0.39
Bur0 × Col0	IVNDFD	1 - 10	4.7633	8 - 16	3.1389 - 7.4543	7.97	11.1	–1.14
Bur0 × Col0	DINAGZ	1 - 14	6.4202	8 - 18	3.1389 - 8.4884	8.98	12.5	–1.50
Blh1 × Col0	ADL/NDF	1 - 28	9.1975	18 - 30	6.5917 - 9.7879	3.85	5.8	–0.27
Bay0 × Shah	KL/NDF	1 - 40	11.9327	32 - 48	9.7705 - 15.4439	3.13	3.6	0.44
Bur0 × Col0	KL/NDF	1 - 66	19.6748	62 - 72	18.3666 - 22.6551	3.80	5.5	0.27
Bay0 × Shah	KL/NDF	1 - 68	23.7365	62 - 70	20.9853 - 24.4610	2.45	2.8	–0.34
Bay0 × Shah	IVNDFD	1 - 70	24.4610	66 - 74	22.9396 - 25.3285	2.83	3.2	0.88
Bur0 × Col0	NDF	1 - 88	26.732	84 - 94	25.5045 - 27.9587	6.70	9.5	–1.09
Bur0 × Col0	IVNDFD	1 - 82	24.860	76 - 86	23.5700 - 26.0615	6.08	8.6	1.19
Bur0 × Col0	DINAGZ	1 - 88	26.732	84 - 94	25.5045 - 27.9587	5.21	7.4	1.10
Blh1 × Col0	NDF	2 - 2	0.8905	0 - 8	0.5926 - 1.7842	4.31	6.4	1.03
Blh1 × Col0	IVNDFD	2 - 0	0.5926	0 - 10	0.5926 - 2.0821	2.35	3.6	–0.67
Blh1 × Col0	DINAGZ	2 - 4	1.1188	0 - 10	0.5926 - 2.0821	4.44	6.6	–1.18
Bur0 × Col0	DINAGZ	2 - 2	0.7410	0 - 6	0.5926 - 4.7931	2.56	3.7	–0.77
Bay0 × Shah	ADL/NDF	2 - 30	9.5702	18 - 36	6.9060 - 11.3716	2.99	3.4	0.27
Bay0 × Shah	KL/NDF	3 - 18	7.7704	10 - 28	6.1737 - 9.7099	4.72	5.3	0.52
Bay0 × Shah	NDF	4 - 4	0.5394	0 - 8	0.0895 - 1.9027	12.1	13.2	2.41
Bay0 × Shah	ADL/NDF	4 - 0	0.0895	0 - 4	0.0895 - 0.5394	7.28	8.1	0.41
Bay0 × Shah	IVNDFD	4 - 4	0.5394	0 - 8	0.0895 - 1.9027	8.68	9.6	–1.74
Bay0 × Shah	DINAGZ	4 - 4	0.5394	0 - 10	0.0895 - 2.4012	8.80	9.7	–2.07
Bur0 × Col0	IVNDFD	4 - 0	0.6414	0 - 8	0.6414 - 4.9648	2.50	3.6	–0.58
Blh1 × Col0	NDF	4 - 0	0.0112	0 - 4	0.0112 - 0.7627	4.31	6.4	0.97
Blh1 × Col0	IVNDFD	4 - 0	0.0112	0 - 8	0.0112 - 1.5137	4.73	7.0	–1.00
Blh1 × Col0	DINAGZ	4 - 0	0.0112	0 - 4	0.0112 - 0.7627	8.44	12.2	–1.51
Bur0 × Col0	KL/NDF	4 - 6	1.9386	2 - 8	1.0738 - 4.9648	4.01	5.8	–0.28
Blh1 × Col0	KL/NDF	4 - 10	1.8892	6 - 12	1.1382 - 4.9160	26.36	33.4	–0.88
Bur0 × Col0	KL/NDF	4 - 52	17.4705	48 - 52	16.2524 - 17.4705	5.42	7.7	–0.40
Bay0 × Shah	NDF	5 - 14	3.4148	12 - 18	2.7319 - 4.7653	4.63	5.2	–1.43
Bay0 × Shah	IVNDFD	5 - 8	1.3726	0 - 14	0.0274 - 3.4148	2.81	3.2	0.92
Bur0 × Col0	NDF	5 - 8	3.4108	6 - 12	2.8630 - 5.4207	8.20	11.4	1.32
Bur0 × Col0	IVNDFD	5 - 8	3.4108	6 - 12	2.8630 - 5.4207	6.61	9.3	–1.11
Blh1 × Col0	NDF	5 - 10	2.5717	6 - 16	1.2584 - 4.1228	5.96	8.8	1.16
Blh1 × Col0	IVNDFD	5 - 10	2.5717	6 - 16	1.2584 - 4.1228	3.38	5.1	–0.87
Blh1 × Col0	DINAGZ	5 - 10	2.5717	6 - 16	1.2584 - 4.1228	3.47	5.2	–0.94
Bay0 × Shah	NDF	5 - 52	15.6035	46 - 58	14.1087 - 17.3150	2.96	3.4	–1.09
Bay0 × Shah	IVNDFD	5 - 60	17.9419	56 - 68	16.7406 - 20.2357	2.39	2.7	0.85
Bay0 × Shah	DINAGZ	5 - 52	15.6035	46 - 58	14.1087 - 17.3150	4.10	4.7	1.28
Bur0 × Col0	KL/NDF	5 - 78	24.0938	74 - 84	22.6696 - 25.6521	5.50	7.8	0.33

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Table 9. Colocalizations between QTL positions and putative candidate genes on chromosome 1. Allelic variation of candidate genes (CDS) between Bur0 and Col0 (NSyn var = non synonymous variation including SNP and Indel).

Gene or RIL	Gene or QTL	Position Mbp	NSyn var
AtLIM1	At1g10200	3.347	0
Bur0 × Col0	NDF	4.763	-
Bur0 × Col0	IVNDFD	4.763	-
SHINE1	At1g15360	5.284	0
Bur0 × Col0	ADL/NDF	5.455	-
AtCCR1 (IRX4)	At1g15950	5.479	0
AtMYB58	At1g16490	5.630	2
Bur0 × Col0	DINAGZ	6.420	-
DOF zinc finger	At1g21340	7.476	0
GRAS	At1g21450	7.509	5
AtMYB3	At1g22640	8.006	-
AtRABG3b	At1g22740	8.050	-
Blh1 × Col0	ADL/NDF	9.198	-
ANAC010 SND3	At1g28470	10.011	-
HDZIP III ATHB9	At1g30490	10.796	-
LOB domain LBD4	At1g31320	11.213	-
ANAC012 SND1	At1g32770	11.865	-
Bay0 × Shah	KL/NDF	11.933	-
At4CL1	At1g51680	19.159	3
HDZip III ATHB15	At1g52150	19.409	1
Bur0 × Col0	KL/NDF	19.675	-
AtMYB75	At1g56650	21.234	14
ABC transporter	At1g59870	22.035	1
bHLH	At1g61660	22.754	1
β-GLU45	At1g61810	22.830	7
β-GLU46	At1g61820	22.835	0
KNAT7 (IRX11)	At1g62990	23.337	1
Bay0 × Shah	KL/NDF	23.736	-
DOF zinc finger	At1g64620	24.007	5
Bay0 × Shah	IVNDFD	24.461	-
Bur0 × Col0	IVNDFD	24.860	-
C3H14 zinc finger	At1g66810	24.928	10
DOF zinc finger	At1g67030	25.017	3
AtMAP70-1	At1g68060	25.511	1
CCCH zinc finger	At1g68200	25.562	0
KNAT2	At1g70510	26.576	1
Bur0 × Col0	NDF	26.732	-
Bur0 × Col0	DINAGZ	26.732	-
AtMIR858a	At1g71002	26.777	0
ANAC030 VND7	At1g71930	27.078	0
C3HC4 zinc finger	At1g72200	27.170	2
C3HC4 zinc finger	At1g72220	27.184	2

in this locus is the AtMYB58 gene, which is a transcriptional activator of monolignol biosynthesis genes in the SND1-mediated network [84]. AtMYB58 binds to an AC element of monolignol biosynthesis genes (PAL1, 4CL1, HCT, C3H1, CCoAOMT1, CCR1). Its over-expression especially greatly increased expression of PAL1, 4CL1, and CCoAOMT1, but a significant, even lesser, increase was also observed for the COMT gene in which AC elements have not been shown [84]. AtMYB58 under- or over-expression have no effect on F5H1 activity, corroborating the fact that this F5H gene is directly regulated by the master genes NST1/SND1 [144]. AtMYB58 also regulated LAC4, but not LAC17 expression [84]. Moreover, a potential natural antisens gene At1g16489 overlaps with the AtMYB58 (At1g16490) gene and it could also be the underlying determinant of the observed QTL. The AtLIM1 gene is orthologous to NtLIM1 [91] and also to ZmLIM1. The SHINE1 gene located close to the QTL position, the DOF-type zinc finger and the GRAS SCARECROW-like1 genes located at the basal part of the QTL support intervals are also possible underlying candidate genes.

3.4.2. Chromosome 1, QTL Mapped at 9 and 12 Mbp

The ADL/NDF QTL detected at 9.2 Mbp in the Blh1 × Col0 progeny and the KL/NDF QTL detected at 11.9 Mbp in the Bay0 × Shahdara progeny, were in close positions with the two master NAC transcription factors SND3 and SND1, respectively. Moreover, the AtMYB3 transcription factor that represses phenylpropanoid biosynthesis gene expression was also located in the upstream area of QTL support intervals. In the QTL support intervals were also located the LBD4 gene preferentially expressed in phloem, the ATHB9 leucine zipper gene, and the AtRABG3b (or RAB7) gene which were likely less probable candidates at these loci than the MYB and the two NAC transcription factors.

3.4.3. Chromosome 1, QTL Mapped at 20 and 25 Mbp

The Bur0 × Col0 and Bay0 × Shahdara QTL that mapped from 19.7 Mbp to 26.7 Mbp had partly overlapping support intervals. Even if it was not possible to definitely affirm the presence of only a single QTL or two distinct QTL in this area, this second hypothesis is the most probable given the structure of QTL positions and support intervals. Several major transcription factors involved in cell wall lignification (AtMYB75, and KNAT7), two DOF-type zinc fingers, the ATHB15 leucine zipper, and one gene involved in monolignol biosynthesis (At4CL1) were in the support intervals of the upstream part of this QTL location. Moreover, KNAT7 and AtMYB75 physically interacts, forming functional complexes that integrate the metabolic flux through the lignin, flavonoid and

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polysaccharide pathways in inflorescence stems [89]. KNAT7 could thus have a simultaneous effect on lignin content and cell wall degradability. The Bur0 × Col0 QTL located in the downstream part of this QTL position first colocalized with the finger transcription factor AtC3H14, a CCCH zinc finger protein which has been shown to activate a lot of the secondary wall phenolics and carbohydrate related genes [95], three other zinc fingers, AtMAP70-1, and with KNAT2. This QTL also colocalized with the master NAC gene VND7 Moreover, the AtMIR858, which targets the master gene AtMYB83, also colocalized with this set of QTL and could be considered a relevant candidate.

3.4.4. Chromosome 2, QTL Mapped 0.8 Mbp

Based on the proposed gene list, which is not exhaustive, no candidate gene was located in the support intervals of these QTL (**Table 10**). Given that no QTL for lignin traits were observed in this position, the underlying determinant should therefore be related to carbohydrate structure. The determinants of the latter were not included in the candidate gene list. However, none of the three cellulose synthase expressed in stems, and no COBRA or FRAGILE FIBER genes were located in QTL support intervals. A survey of genes located under these QTL support intervals shows the presence, between At2g02260 and At2g05620, of 384 protein-coding genes out of which 64 encode proteins of unknown function.

3.4.5. Chromosome 2, QTL Mapped at 9.5 Mbp

Three putative candidate genes with established involvement in lignified tissue assembly colocalized with the ADL/NDF QTL detected in the Bay0 × Shahdara progeny. The continuous vascular ring (COV1) defective mutant displays an increase in vascular tissue development in the stem interfascicular regions [102]. The LCV3 (like-COV-3), which is located in close upstream position to COV1, is also supposed to be involved in stem vascular tissue pattern formation. The C3HC4 zinc finger was the third possible candidate gene underlying this QTL.

3.4.6. Chromosome 3, QTL Mapped at 7.8 Mbp

Only one QTL for KL/NDF was observed on chromosome 3, in the Bay0 × Shahdara progeny. The AtCAD-C and two 4CL genes (possibly) involved in the constitutive monolignol biosynthesis pathway were located in the upstream part of the QTL, together with an ABC transporter in the downstream part (**Table 11**). In addition, a DOF-type zinc finger and the AtBAB7b GTPase genes were also located in the QTL support interval.

3.4.7. Chromosome 4, QTL Mapped at 0.3 Mbp

Eight QTL originating from the three progenies and related to cell wall content, lignin content and cell wall digestibility colocalized in the upstream part of chromosome 4, centred on the position 0.3 Mbp (**Table 12**). Only two cell wall related candidate genes were shown for the upstream QTL, with the LBD30 and XSP1 genes. In addition, the AtMIR165b miRNA, which was also located in the central part of the QTL support interval, targets HDZIP genes involved in vascular tissue differentiation, including PHV, PHB, REV, ATHB-8, and ATHB-15. Variation in AtMIR165b expression of efficiency could therefore explain differences in lignin content and cell wall degradability. However, another hypothesis could be considered in the search for the underlying determinant of these QTL. The FRIGIDA (flowering locus A, FRI, At4g00650) gene, which encodes a major determinant of natural variation in Arabidopsis flowering time, is located in the central part of the QTL support intervals at position 0.269 Mbp. It could therefore be assumed that the differences observed in lignin traits might result from differences in earliness, duration of stem growth, and consequential different lignification between parental ecotypes. Many early flowering accessions carry loss-of-function mutation in the FRI alleles, including the reference ecotype Col0 [145], and Bay-0

Table 10. Colocalizations between QTL positions and putative candidate genes on chromosome 2.

Gene or RIL	Gene or QTL	Position Mbp
Blh1 × Col0	IVNDFD	0.593
Bur0 × Col0	DINAGZ	0.741
Blh1 × Col0	NDF	0.890
Blh1 × Col0	DINAGZ	1.119
COV LCV3	At2g18460	8.001
COV1	At2g20120	8.687
C3HC4 zinc finger	At2g20650	8.903
Bay0 × Shah	ADL/NDF	9.570

Table 11. Colocalizations between QTL positions and putative candidate genes on chromosome 3.

Gene or RIL	Gene or QTL	Position Mbp
AtRAB7b	At3g18820	6.484
AtCAD-C	At3g19450	6.745
At4CL5	At3g21230	7.448
At4CL2	At3g21240	7.454
ADOF2 zinc finger	At3g21270	7.475
Bay0 × Shah	KL/NDF	7.770
ABC transporter	At3g25620	9.313

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Table 12. Colocalizations between QTL positions and putative candidate genes on chromosome 4. Allelic variation of candidate genes (CDS) between Bur0 and Col0 (NSyn var = non synonymous variation including SNP and Indel, na = missing data).

Gene or RIL	Gene or QTL	Position Mbp	NSyn var
Blh1 × Col0	NDF	0.011	-
Blh1 × Col0	IVNDFD	0.011	-
Blh1 × Col0	DINAGZ	0.011	-
Bay0 × Shah	ADL/NDF	0.089	-
LBD30	At4g00220	0.090	0
XSP1	At4g00230	0.094	4
AtMIR165b	At4g00885	0.370	0
Bay0 × Shah	NDF	0.539	-
Bay0 × Shah	IVNDFD	0.539	-
Bay0 × Shah	DINAGZ	0.539	-
Bur0 × Col0	IVNDFD	0.641	-
Blh1 × Col0	KL/NDF	1.889	-
Bur0 × Col0	KL/NDF	1.939	-
AtMIR397a	At4g05105	2.626	15
AtCCoAOMT1	At4g34050	16.311	0
AtCAD-D	At4g34230	16.387	0
AtMYB32	At4g34990	16.663	1
bZIP19	At4g35040	16.680	0
HCA	unknown	16.795	-
XCP1	At4g35350	16.810	0
AtF5H1	At4g36220	17.137	0
Bur0 × Col0	KL/NDF	17.470	-
AtPRX51	At4g37530	17.632	na
AtMYB4	At4g38620	18.054	0

which carry the same recessive allele as Col0. Conversely, Shahdara FRI allele was considered fully functional [30,146]. The two ecotypes Bur0 and Blh1 also have a functional FRI allele ([147]; Camilleri *et al.*, unpublished data). In all cases, alleles increasing cell wall degradability were shown in ecotypes with loss-of-function FRI allele (Bay0 and Col0). A candidate gene or miRNA effect strengthened by differences in growth duration could also be hypothesized.

3.4.8. Chromosome 4, QTL Mapped at 1.9 Mbp

Even if the two KL/NDF QTL mapped at position 1.9 Mbp on chromosome 4 had partly overlapping positions with several QTL at the 0.3 Mbp position, they likely corresponded to a different underlying determinant. Moreover, the KL/NDF QTL observed in the Blh1 × Col0 progeny had the highest Lod (26.4) and R2 (33.4%) values out of all observed QTL. No candidate genes were shown colocalizing with these two QTL. However, the AtMIR397a was located at the basal part of the QTL support intervals. The AtMIR397a encodes a microRNA that targets several laccase family members. In Arabidopsis, two double mutants AtLAC4-AtLAC17 had a reduced Klason lignin content by 20% and 40%, respecttively [12].

3.4.9. Chromosome 4, QTL Mapped at 17.5 Mbp

The isolated KL/NDF mapped at this position on chromosome 4 colocalized with several lignin-related genes, including genes involved in monolignol biosynthesis (AtCCoAOMT1, AtF5H1, AtCAD-D) and the AtPRX51 peroxidase. Colocalization was also shown with one bZIP, the XCP1 serine protease, and the HCA gene of which the position is only approximate. The latter were all putatively involved in tissue assembly and patterning.

3.4.10. Chromosome 5, QTL Mapped at 2.5 and 3.4 Mbp

On the upstream part of chromosome 5, all seven detected QTL have overlapping support intervals. It could not definitely be concluded whether one or two genetic determinants corresponded to the most likely situation. This QTL area is typified by the fact that colocalizations brought together QTL for cell wall content and degradeability, but no QTL for lignin content. The AtMYB46 gene located at the downstream part of QTL support intervals (**Table 13**), is, along with AtMYB83, the closest ortholog of EgMYB2, a positive regulator of lignification [148]. This AtMYB46 gene could have been a relevant candidate. However, variations driven by such a determinant might have also induced differences and QTL for lignin content. The two NAC genes VNI1 and VNI2 are repressors of xylem vessel development in roots and aerial organs [86]. The ERF/AP2 SHINE2, AtRAB8c, and AtBAG3 genes could also be considered as inducing variations in the assembly of the different lignified tissues. The laccases and peroxidase were less probable candidates as they also might have induced differences in lignin content. In addition, the two AtMIR166, which were located in central part of QTL support intervals, could also be the underlying determinants.

3.4.11. Chromosome 5, QTL Mapped at 17.0 Mbp

Considering only one genetic determinant for the three QTL only detected in Bay0 × Shahdara progeny at the basal part of chromosome 5, one NAC, one CCCH zinc finger, one bHLH gene, and one member of the ROP GTPase family, could be assumed as the main candidates. In addition the two AtMIR166e and AtMIR166f also colocalized with the QTL position. The AtHCT gene was

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Table 13. Colocalizations between QTL positions and putative candidate genes on chromosome 5. Allelic variation of candidate genes (CDS) between Bur0 and Col0 (NSyn var = non synonymous variation including SNP and Indel).

Gene or RIL	Gene or QTL	Position Mbp	NSyn var
AtLAC10	At5g01190	0.072	-
AtLAC11	At5g03260	0.777	-
AtRAB8c	At5g03520	0.883	-
Bay0 × Shah	IVNDFD	1.372	-
AtLAC12	At5g05390	1.595	-
AtPRX53	At5g06720	2.077	-
AtBAG3	At5g07220	2.265	-
Blh1 × Col0	NDF	2.572	-
Blh1 × Col0	IVNDFD	2.572	-
Blh1 × Col0	DINAGZ	2.572	-
AtMIR166c	At5g08712	2.839	0
AtMIR166d	At5g08717	2.840	0
AtNAC082 VNI1	At5g09330	2.892	1
Bur0 × Col0	NDF	3.411	-
Bur0 × Col0	IVNDFD	3.411	-
Bay0 × Shah	NDF	3.415	-
ERF SHINE2	At5g11190	3.566	1
AtMYB46	At5g12870	4.063	0
AtNAC083 VNI2	At5g13180	4.196	1
KANADI1	At5g16560	5.407	0
Bay0 × Shah	NDF	15.605	-
Bay0 × Shah	DINAGZ	15.604	-
AtMIR166e	At5g41905	16.793	-
AtPRX64	At5g42180	16.853	-
CCCH zinc finger	At5g42820	17.170	-
AtMIR166f	At5g43603	17.535	-
Bay0 × Shah	IVNDFD	17.942	-
AtROP7	At5g45970	18.644	-
bHLH	At5g48560	19.684	-
AtHCT	At5g48930	19.836	-
Bur0 × Col0	KL/NDF	24.093	-
AtLAC17	At5g60020	24.168	0
HDZip III IFL1	At5g60690	24.397	4
DOF zinc finger	At5g60850	24.804	0
ANAC101 VND6	At5g62380	25.051	0
AtROP11	At5g62880	25.237	0
HCA2 zinc finger	At5g62940	25.257	0
AtMIR166g	At5g63715	25.522	0

a less possible candidate as it was located at the downstream part of the IVNDFD QTL support interval.

3.4.12. Chromosome 5, QTL Mapped at 24.1 Mbp

Several candidate genes were closely located to the position of the isolated KL/NDF QTL on chromosome 5, including the master NAC VND6, the HDZip IFL1, the HCA2 DOF-type zing finger and another DOF-type zinc finger, and the AtROP11 RAC GTPase gene. In addition, the AtMIR166g was located at the basal part of the QTL support interval.

3.5. Allelic Variation of Candidate Genes between Bur0 and Col0 Ecotypes

Allelic variation has been investigated in the coding sequence (CDS) of 43 candidate genes located in the support intervals of seven QTL shown in the Bur0 × Col0 progeny on chromosomes 1 (23 genes), 4 (9 genes), and 5 (11 genes). Among these genes, no SNP or Indel allelic variation was shown in 14 genes. In the 29 genes with allelic variation, 93 synonymous SNP, 56 non synonymous SNP and 6 Indels were shown. However, three genes (GRAS SCARECROW-like At1g21450, AtMYB75 At1g56650, and AtC3H14 zinc-finger At1g66810) exhibited most of the observed variation with 41 synonymous SNP, 27 non synonymous SNP, and 2 Indels (**Tables 9**, **12** and **13**).

3.5.1. Chromosome 1, Bur0 × Col0 QTL Mapped at 5.5 Mbp

Among several candidate genes located in the support interval of lignin-related QTL, the CCR1 gene was a priori the most probable. However, no SNP or Indel were shown in the two CDS between Bur0 and Col0 (**Table 9**), nor in the 5' areas (data not shown). Conversely, a non synonymous SNP (A/C substitution in position 836) and two glutamine deletions (ACAACA deletion in position 912) were shown in the MYB58 gene sequences. The QQ deletion was just flanking the right side of the R2R3 motif WFKHLESELGLEExDNQQ [149] of the MYB58 gene. Five non synonymous SNP were also shown in the GRAS SCARECROW-like gene in positions 1111, 1133, 1433, 1434, and 1633. In addition, the GRAS SCARECROW-like genes in Bur0 and Col0 exhibited large sequence extra variation with 25 synonymous SNP in the CDS.

3.5.2. Chromosome 1, Bur0 × Col0 QTL Mapped at 20 and 25 Mbp

Seventeen candidate genes were located in the support intervals of lignin-related QTL, with at least two and possibly three QTL positions. In the upstream part of the region, three non synonymous SNP were shown in the

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4CL1 CDS (T/C, A/G, and C/A in positions 159, 517, and 1484, respectively). The AtMYB75 gene was extremely different between Col0 and Bur0 (**Table 9**). More especially, a G insertion in position 200 induced a modified ORF and a stop codon with a protein reduced to 68 amino acids in comparison with the 248 amino acids of the normal protein. The AtMYB75 was shown to act as a repressor of the lignin pathway [89]. The AtMYB75 inactivation in Bur0 would be in agreement with the increasing allelic effect of the KL/NDF lignin QTL located in close position to the latter gene. While the CDS of the *β*-glucosidase *β*-GLU46 were very similar in Bur0 and Col0 with only one synonymous SNP, the opposite situation was observed in the *β*-GLU45 *β*-glucosidase with two synonymous and seven non synonymous SNP between Bur0 and Col0 likely inducing different conformations and efficiencies of the two proteins. In a close position to IVNDFD and NDF QTL, the gene encoding the AtC3H14 zinc finger protein was also very different in the two ecotypes with nine non synonymous SNP and one Indel inducing a K/EF change in the protein (AAT insertion in position 172). Similarly to the *β*-GLU45 gene, these changes likely induced different conformations and efficiencies in the Bur0 and Col0 AtC3H14 zinc finger proteins. Because the AtC3H14 has been shown to activate a lot of the secondary wall phenolics and carbohydrate related genes [95], and because alleles from Bur0 increased IVNDFD and NDF content, it should be considered that the AtC3H14 has a lower efficiency resulting in less NDF content of likely higher degradability.

3.5.3. Chromosome 4, Bur0 × Col0 QTL Mapped at 0.3 Mbp

Only two candidate genes were shown in the support interval of QTL located in the upstream part of chromosome 4. Only the XSP1 exhibited CDS variation with two non synonymous SNP in positions 545 and 821 (A/G and T/A changes) inducing N/I and V/D amino acid changes. These changes were likely to induce changes in protein conformations and efficiencies. In addition, no changes were shown in the AtMIR165b sequence between Col0 and Bur0 (**Table 12**).

3.5.4. Chromosome 4, Bur0 × Col0 QTL Mapped at 1.9 Mbp

In this position corresponding to a KL/NDF QTL, only the AtMIR397a was considered as a candidate gene. AtMIR397a is a microRNA that targets several laccase family members. The two corresponding sequences were highly different between Col0 and Bur0 with 14 base substitutions in a 109 bp long cDNA. These substitutions did not affect the 21 bp long mature MIR sequence, but likely affected the stem-loop secondary structure and thus the subsequent processing of the hairpin loop.

3.5.5. Chromosome 4, Bur0 × Col0 QTL Mapped at 17.5 Mbp

Only one candidate gene located in the support interval of the KL/NDF QTL shown in the basal part of chromosome 4 exhibited non synonymous SNP variation. In position 729 of the AtMYB32 gene, a T/A CDS substitution induced a Y/H amino acid change, with possible effects on protein conformations and efficiencies.

3.5.6. Chromosome 5, Bur0 × Col0 QTL Mapped at 3.4 Mbp

The AtMYB46 gene, whose role in Arabidopsis stem lignification has been clearly established, was not considered to be the most probable candidate for the cell wall degradability QTL located in the upstream part of chromosome 5 because the lack of colocalizations with lignin QTL (**Table 13**). No SNP or Indel were indeed shown for AtMYB46 CDS between Col0 and Bur0. Moreover, the two alleles were also identical in their 5' and 3' sequences (data not shown). The cell wall degradability QTL colocalized in this location with cell wall (NDF) content QTL. Observed variation in cell wall degradability would be free of variation in lignification intensity, but negatively related to cell wall content. Among the candidate genes located in this area, CDS allelic variation were only shown for the SHINE2 gene with an AAG deletion in Bur0 (position 483) inducing a change from KE to K amino acids, with the loss of a glutamic acid. The SHINE2 ERF/AP2 gene was considered to be an upstream transcriptional regulator of both master and secondary target genes involved in the biosynthesis of cell wall phenolic and carbohydrate components. Its higher expression induced higher cellulose and hemicellulose contents [127]. Because allele increasing NDF content originated from Bur0, a higher efficiency of the protein with the E deletion should be considered.

3.5.7. Chromosome 5, Bur0 × Col0 QTL Mapped at 24.1 Mbp

Five putative candidate genes out of the six considered did not exhibit any SNP or Indel in their corresponding CDS, and similarly for the colocalizing AtmiRNA166g. Only one non synonymous SNP in the CDS of the HDZIPIII IFL1 REVOLUTA gene was shown with a T/C substitution in position 1874 inducing an F/S amino acid change. Little variation was observed for considered genes between Bur0 and Col0 in this area of chromosome 5.

4. CONCLUSIONS

While QTL investigations have been reported in numer-

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ous plant species, the underlying candidate genes have rarely been identified. Moreover, it is not known whether a QTL most often corresponds to one or several physically linked genes, and in case of QTL colocalization for different related traits, whether there are correlated or pleiotropic actions of only one gene, or several genes with close physical positions, each affecting one or a subset of trait variation.

Based on the set of 133 putative candidates considered, colocalizations with QTL were observed for 70 genes in the three RIL progenies, thus corresponding to 52% of colocalizing genes while the sum of QTL support intervals corresponded to 30% of the Arabidopsis genome (35 out of 115 Mbp, excluding centromer areas). Out of the 16 genes of the monolignol pathway, 9 were found colocalizing with QTL, including 4CL1/2/5, HCT, CCoAOMT1, F5H, CCR1, AtCAD-C/D, but no PAL which are the entry genes of the monolignol pathway. Considering genes involved in monolignol polymerization, three peroxidases out of six, and four laccases out of six, were shown colocalizing with QTL. Colocalizations between genes and QTL were also shown for transcription factors of the NAC and MYB families with six NAC out of ten and three MYB out of eight in colocalizing positions. Similarly, six CCCH/C3HC4 zinc finger genes out of ten and two KNAT genes out of three were in colocalizing positions. For genes putatively involved in tissue patterning, five DOF-type zinc fingers out of six and three HDZip out of six colocalized with QTL. DOF zinc finger, NAC, C3H zinc finger, HDZip, and MYB were in decreasing orders the families with members more frequently associated with QTL positions. Similarly, eight AtMIR out of the 14 considered also colocalized with QTL, including five miRNA166 out of seven, one miRNA165, one miRNA397, and the miRNA858a. Colocalizations were also shown for genes in families with one or a few members (LIM, HCA, COV, ERF SHINE, MAP, GRAS SCARECROW-like and KANADI1 genes). No colocalization was observed for SHATTERPROOF gene likely corroborating its major involvement in silique lignification. Moreover, no WRKY transcription factors colocalized with QTL, and only two ROP out of seven, and two LBD out of seven were in colocalizing positions This likely indicates that these families are possibly not or little involved in the regulation of lignified tissue assembly resulting in degradability differences.

As a tentative conclusion regarding putative determinants and based on observed colocalizations in the three RIL progenies and allelic variation between Bur0 and Col0 CDS, genes underlying the cell wall related QTL are more probably transcription factors regulating cell wall phenolic component biosynthesis. In addition, they also are more likely to be genes involved in lignified tissue patterning than genes involved in monolignol biosynthesis and polymerization. The MYB32, MYB58, MYB75, GRAS SCARECROW, AtC3H14 zinc finger, SHINE2, IFL1, and the AtMIR397a were shown to be very plausible candidates. However, this does not obscure the fact that the true candidates are possibly also outside the considered list, especially because QTL support intervals encompassed genes of still unknown function. Moreover, for application to grasses, the lack of secondary cambium in grasses and the role of *p*-hydroxycinnamic acid in grass cell walls might be taken into account. Many cell wall genes are specific to grasses, and orthologs of genes involved in secondary cambium assembly of dicotyledonous plants might have an originnal function in grasses or do not exist.

The search for candidate gene and allele CDS variation is a first step towards functional validation of the highlighted candidates underlying QTL. In addition to whole allele sequencing, it is worth investigating mutants of the considered candidates that have not yet been studied. Positional cloning, which is a long and tedious approach, will probably be inescapable for final validations. In any case, investigating QTL for cell wall degradability and their underlying determinants is an essential key approach when it comes to improving forage feeding value and saccharification yield in bio-ethanol production.

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