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C. R. Biologies 327 (2004) 467–479
COMPTES RENDUS BIOLOGIES

Plant biology and pathology / Biologie et pathologie végétales

Genetic and molecular basis of grass cell-wall biosynthesis and degradability. III. Towards a forage grass ideotype

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Received 30 January 2004; accepted after revision 23 March 2004

Available online 13 May 2004

Presented by Michel Thellier

Abstract

Lignification of cell walls is the major factor controlling the digestibility of forage grasses. Thus far, from QTL analysis, about 15 locations involved in cell-wall lignification or digestibility have been identified in the maize genome, many of which colocalise with QTLs involved in corn borer susceptibility. Genetic diversity for enhancing cell-wall digestibility in maize must be identified in novel germplasm, but genetic engineering is also a relevant way both to design specific cell-wall characteristics for improved digestibility and to identify genes involved in these traits for further discovery of alleles of interest in grass germplasm. ***To cite this article: J. Ralph et al., C. R. Biologies 327 (2004).***

Résumé

Bases génétiques et moléculaires de la biosynthèse et de la biodégradabilité des parois de graminées. III. Les bases de la construction d'un idéotype de graminée fourragère. La lignification et la structure des parois sont les deux facteurs essentiellement liés à leur digestibilité. Une quinzaine de régions impliquées dans ces caractères a été mise en évidence après recherche de QTL chez le maïs, dont une partie significative colocalise avec des QTL de tolérance à la pyrale. Les ressources génétiques pour l'amélioration de la digestibilité sont à rechercher au sein du matériel génétique original. Mais la transgenèse est aussi une voie majeure, autant pour construire des ressources génétiques d'intérêt que pour mettre en évidence les gènes impliqués et rechercher ensuite des allèles d'intérêt dans le matériel classique. ***Pour citer cet article : J. Ralph et al., C. R. Biologies 327 (2004).***

Keywords: grass; lignin; cell wall; digestibility; genetic resources

Mots-clés : graminées ; lignine ; paroi ; digestibilité ; ressources génétiques

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1631-0691/$ – see front matter
doi:10.1016/j.crvi.2004.03.004

1. Introduction

Forage grasses serve as a basis of ruminant and herbivore nutrition. Although forages contain almost the same amount of gross energy as do cereal grains per unit of dry matter, the energy value of forage grasses is lower and much more variable, ranging approximately from 33% (wheat straw) to 70 (silage maize) or 80% (leafy ray-grass) of maize grain value. The difference in energy value between grain and forage results from the high content of cell wall in forage plants, and to the limited digestion by ruminant animals of this fiber fraction by microorganisms in the rumen and, to a lesser degree, in the large intestine. Below are documented large inter-species variations; significant genetic variations were also demonstrated within species. On a genetic basis, cell-wall digestibility of forage maize had a variation range of about 15 to 25 percentage points, about an average value close to 50% [1]. The quantitative importance of lignins in the cell wall, their variable structure, and a variety of cross-linkages between cell-wall components all have variable depressive effects on cell-wall carbohydrate degradation by microorganisms [2]. Based on European or Northern America experiments, genetic variation in cell-wall digestibility of maize silage was also shown to impact young bull and dairy cow performance, even if maize was not the only constituent of the diet. All other factors being equal, when comparing hybrids with poor vs good cell-wall digestibility in dairy cows, fat corrected milk yields differed from 1 to 3 kg among hybrids, and differences in average daily gains of young bulls reached 100 g per day [1]. Cell-wall digestibility is therefore recognized as a major target for improving the feeding value of grasses (and forage crops).

An ideotype of a forage grass can be drawn according to both (*i*) the actual understanding of, and current hypotheses on, the lignin pathway and cell-wall cross-linking, (*ii*) the actual knowledge of genetic determinants involved in plant lignification and cell-wall digestibility, and (*iii*) the agronomic, economic, and environmental contexts of forage plant cropping. For cattle feeding, a forage plant ideotype might have both highly digestible cell walls and a good friability of the plant tissues in order to provide high ingestibility. In maize, a starch content nearing 30% is considered optimal in order to avoid negative interactions between cellulolytic and amilolytic flora of the rumen, and acidosis risks. In perennial leafy grasses, a high soluble carbohydrate content is related to the energy content in the plant. Good standability of the plant is essential, and standability is related to cell-wall lignin and carbohydrate content and structure, but also to the correlated mechanical characteristics (flexibility) of the stalk, and to the quality of the plant's root anchorage.

In two previous papers [2,3], an overview of the biochemical, genetic and molecular determinants of grass cell-wall biosynthesis was presented. The objective of this text is then to assess the best options toward breeding grasses for improved digestibility, based on a search for genome locations involved in these traits through QTL analysis, and based on genetic engineering investigations in order to design specific cell-wall characteristics for improved digestibility and to identify genes involved in these traits for further discovery of alleles of interest in grass germplasm.

2. QTLs and candidate genes involved in cell-wall biosynthesis and digestibility

Although QTL investigations have been numerous in maize, only a limited number of studies have been devoted to forage maize and its feeding value. Lübberstedt et al. [4,5] published the first QTL analysis devoted to quality traits, but investigated QTLs were related to whole plant digestibility. QTLs for cell-wall digestibility and lignification traits in maize were further investigated in two RIL progenies by Méchin et al. [6] and Roussel et al. [7]. Preliminary data were also available from unpublished results from the INRA–ProMaïs network. Moreover, QTLs for lignin content were given by Cardinal et al. [8] in a progeny developed for corn borer tolerance studies. The synthesis of these data showed that five major QTL clusters, located in possible order of importance according to both their LOD values and their percentage of explained phenotypic variation, were found in bins 6.06, 3.05/06, 1.03, 8.05, and 9.02 (Table 1). Additional locations or other clusters were also involved in cell-wall digestibility and lignification traits, located in nine other bins. In most cases, QTLs for lignin content and cell-wall digestibility were found in the same location. This could partly be a bias of the enzymatic solubility used for the cell-wall digestibility estimates. Enzy-

Table 1
Putative colocalizations, between (*i*) QTL identified for cell-wall lignin content, esters pCA and FA, or digestibility, (*ii*) QTL identified for European corn borer tolerance and (*iii*) genes of the lignin pathway.
– QTL for KL/NDF, ADL/NDF, Esters pCA and FA, DINAG or DINAGZ are given from studies in 4 RIL progenies: (1) 100 RILs F2 × Io after RIL per se value (RIL) and top cross experiments (TC) in Méchin et al. [6], (2) 131 RILs F288 × F271 after RIL and TC experiments in Roussel et al. [7] and Fontaine et al. [96], (3) preliminary results from 252 RILs F838 × F286 obtained in the INRA–ProMaïs network after RIL experiments, (4) RIL per se experiment, and stalk lignin content in Cardinal et al. [8] from 200 RILs in B73 × B52 ($R^2 > 5\%$), after TC experiments.
– QTLs for European corn borer tolerance (ECB, 1ECB first generation, 2ECB second generation) are given from studies in 6 RIL progenies: (1) Schön et al. [97], (2) Bohn et al. [98], (3) Cardinal et al. [99], (4) Papst et al. [100], (5) Jampatong et al. [101], (6) Krakowsky et al. [102].
– Gene localization is given according to public and published data [1,9,10]

Bin	Evaluation type	RIL progeny	Lignification and cross-linking traits	European corn borer tolerance	Gene localization
1.02	RIL	1, 2	KL/NDF, DINAGZ	2ECB (*1*)	–
1.03	RIL	1, 2, 3	ADL/NDF, KL/NDF, DINAGZ	–	–
1.07	RIL	4	ADL	2ECB (*1*), ECB (*2*, *4*)	4CL2, F5H, CCR1
2.04	RIL	2	ester FA	ECB (*3*)	–
2.08	RIL, TC	2, 4	ADL/NDF, DINAGZ, ADL	2ECB (*1*), ECB (*3*, *6*)	px1
3.01	RIL	4	ADL	ECB (*3*)	–
3.03/04	RIL	2	ester FA	–	–
3.05/06	RIL, TC	2, 4	ADL/NDF, DINAGZ, ADL	2ECB (*1*), ECB (*3*)	myb2
3.08	RIL	2	ester pCA	–	–
4.05	RIL	1	ADL/NDF	–	CaldOMT, PAL3
4.08	RIL, TC	1, 2	DINAG, ester FA	–	–
4.09/10	RIL	1, 2	ADL/NDF, DINAGZ, ester pCA	ECB (*4*)	–
5.03	RIL	4	ADL	ECB (*2*, *4*, *6*)	4CL1, CAD2, px13
6.01	RIL, TC	2	ADL/NDF, DINAGZ	–	CCoAOMT1
6.04	RIL	2	ester pCA	–	–
6.05	RIL	4	ADL	ECB (*6*)	–
6.06	RIL, TC	1, 2	ADL/NDF, KL/NDF, DINAGZ	–	–
7.02/03	RIL	1	DINAG	ECB (*3*, *6*)	–
8.05	RIL, TC	3	ADL/NDF, KL/NDF, DINAGZ	ECB (*2*, *4*)	myb MRP1
9.01	RIL	2	ester pCA	ECB (*3*)	–
9.02	RIL, TC	1, 2	ADL/NDF, KL/NDF, DINAGZ	2ECB (*5*, *6*)	CCoAOMT2
10.05	RIL	4	ADL	2ECB (*1*, *6*)	C4H

matic solubilities, and cell-wall-based digestibilities, were indeed shown to be more related to lignin content than cell digestibilities estimated in animals [1]. Conflicting situations in maize breeding for cell-wall digestibility will probably result from the frequent colocalisations between QTLs involved in wall lignification and digestibility, and QTLs for European corn borer tolerance (Table 1). Out of 22 locations involved in wall digestibility and/or lignin content, 14 were also described as involved in *Ostrinia nubilalis* tolerance (tunneling length or stalk damage rating). Today, it cannot be dismissed that genotypes with high cell-wall digestibilities will be more susceptible to pest damage. The genes underlying QTLs are not yet really known, despite information is available based on public or published data [1,9,10]. Each known gene of the maize lignin pathway was thus found co-localizing with QTLs. But important locations such as bins 1.03 and 6.06, in which no colocalizations with corn borer tolerance QTLs were found, remain without any (public) candidate genes. However, each maize bin has on average 27 Mbp (2500 Mbp/91 bins), corresponding approximately to 20% of the *Arabidopsis* genome, and colocalization in the same bin can not be considered as a definitive direct linkage of the traits, especially as genes of the lignin pathway are likely gathered in clusters.

QTLs for lignin content and cell-wall digestibility of plant leaves and sheaths were also investigated in the progeny of an interspecific backcross with Lolium in a Lolium × Festuca progeny by Marhadour [11]. Two markers, originating from fescue, explained 9.0

and 5.5% of the lignin content variation, respectively. Another marker originating from ryegrass explained 4.5% of whole plant digestibility, but only 1.1% of cell-wall digestibility variation. Today, seemingly no more QTL analysis of cell-wall digestibility or lignification has been investigated in grasses.

3. Genetic resources in forage breeding for improved cell-wall digestibility

Forage plant germplasm developed during prior decades allowed major improvements in yield, yield regularity, plant rusticity, stalk standability, and disease or pest tolerance. However, feeding value was often not considered for registration. Today, leaf pliability is considered in fescue, and since 1998, whole plant digestibility is considered in maize (in France). Significant drift of maize registered hybrids towards lower in vivo digestibility values were observed in the last two or three decades [1,12] in France, but not in the USA [13], probably because of different evolution patterns of the maize germplasm. In Europe, most of modern forage maize hybrids are based on a germplasm previously bred and/or used in grain maize hybrids. It is then very likely that alleles allowing good digestibility and good friability were eliminated during breeding for stalk standability and breakage resistance. The search for highly digestible and ingestible maize (or any forage plant) will require a new investigation of (old) genetic resources that are not currently used. They may have been neglected because they lead to poor-yielding genotypes (lower additive value, and lower heterotic pattern), which are often susceptible to lodging and stalk rotting. However, there is no evidence of an absolute or definitive linkage between these poor agronomic traits and the feeding value in maize [14] or in other grasses [15]. However, because there is obviously a considerable gap in agronomic value between these old lines (or resources) and elite modern lines (or resources), specific strategies of introgressing feeding value traits in elite germplasm must be considered. One way is to identify QTLs or genes of interest for feeding value prior to any breeding effort, allowing either carefully targeted marker assisted selection (MAS) based on flanking SSR markers, with a very small modification of the backcrossed elite lines, or more efficiently, the selection of favorable alleles through SNP (single nucleotide polymorphism) or INDEL (insertion – deletion polymorphism) variation. Limits of such a breeding methodology are related to the possibility of insufficient linkages between markers and traits, to marker associated gene(s) accounting for too little of the trait variation, or to epistasis or complex relationships between the selected gene(s) and the genetic background of the old resource. Another methodology is to develop specific donor lines of both high cell-wall digestibility and correct agronomic value based on usual phenotypic breeding for all traits in recurrent crosses between elite lines and old resources. Limits of this breeding approach are then related to the management of the high number of plants, topcrosses and cell-wall digestibility estimates, to the necessity of having successive cycles of breeding, and to the risk of eliminating alleles of interest for cell-wall digestibility when selecting for agronomic value. Moreover, this approach needs a step more in the breeding process of elite lines, as these donor lines have to be crossed with elite germplasm to produce cell-wall digestibility improved elite lines. However, QTL or SNP investigations, followed by MAS, should likely be very efficient with such donor lines whose genetic backgrounds will be closer to elite lines than to old resources, except for areas of interest in cell-wall digestibility.

Another relevant approach toward breeding forage grasses of higher digestibility and ingestibility is to devise specific genetic resources through genetic engineering of the lignin pathway. Genetically modified plants could then directly be used for feeding, but transgenesis is also a particularly efficient way to uncover the more relevant targets for classical investigation of genetic resources, and breeding of normal genotypes. Extensive reviews on genetic engineering in the lignin pathway have been recently published [16–22]. Highly variable consequences on lignin content and structure have often been observed in altered transgenic plants, and these inconsistencies highlight the necessity to further evaluate the effects of genes and promoters used, especially because of the interaction with the spatio-temporal deposition in each lignified tissue. However, results clearly established the more or less efficient antisense or silencing strategies for reducing lignin content and/or modifying lignin structure or cell-wall phenolics of plants, in turn altering cell-wall digestibility. Nevertheless, very few

papers were devoted to transgenesis in monocotyledons. Only two papers have seemingly been published on maize [23,24], and one on fescue [25]. In accordance with data on cell-wall biosynthesis previously developed [2,3], the objective of genetic engineering for improved feeding value in grasses should be (*i*) to decrease lignin content, but limit the decrease to avoid detrimental effects on agronomic traits, (*ii*) to alter the lignin composition, with possible concomitant effects on lignin–polysaccharide cross-linking (driving S/G towards a higher value could be investigated even though it has been shown that syringyl/guaiacyl differences, per se, have no effect on digestibility [26]), (*iii*) to drive the spatio-temporal regulation of lignification in order to optimize lignification only in places where it is necessary for water and nutrients conduction, and stalk standability, i.e. produce parenchyma cells that are not lignified, (*iv*) to reduce cross-linking between both lignin and hemicelluloses, and within hemicelluloses chains and/or (*v*) to drive the cell-wall carbohydrates biosynthesis and their relationships with lignins and hydroxycinnamic acids so as to obtain a compromise between friability, accessibility to microorganisms, and mechanical properties of agronomic interest. Not all of the information on favorable lignification patterns in leaves or stalks is yet known and available. Because numerous QTLs involved in cell-wall lignification or digestibility remain without candidate genes, or without validated candidate genes, targets for genetic engineering towards a forage plant ideotype are nowadays only known for a sub-sample of all genes of potential interest. Nevertheless, favorable QTLs can be used as genetic resources and introgressed through marker assisted selection in elite lines, even if the underlying determinants are not understood.

4. Target genes for genetic engineering of cell-wall digestibility

The alteration of early steps in phenylpropanoid metabolism (PAL, phenylalanine ammonia-lyase; C4H, cinnamate 4-hydroxylase), which are involved in other important processes, besides lignification, in plants, was considered likely to lead to too many adverse pleiotropic effects to be usefully considered for cell-wall digestibility improvement [1,27,28]. Conversely, CCR (cinnamoyl CoA reductase) and CAD (cinnamyl alcohol dehydrogenase), which "function after all possible branch points in the pathway", have been then considered as potentially suitable targets [27]. However, genes of the lignin pathway appeared to belong to multigene families, even though individual genes could have distinct metabolic roles. Engineering in early steps of the lignin pathway should probably be re-considered, as a different metabolic flux may exist for each specific pattern. Anterola and Lewis [29] indeed considered "there may be distinct phenylalanine pools for specific purposes [...] due to the existence of distinct and coordinated metabolic networks". Down-regulation of PAL and C4H in tobacco led to plants probably no more disturbed than plants down-regulated in other genes of the lignin pathway [19,29]. In addition, *Arabidopsis* At4CL1 and At4CL2 (4-coumarate-CoA ligase) were considered to be involved in lignification, whereas At4CL3 was considered to function in flavonoid biosynthesis [30], and similarly for Pt4CL1 and Pt4CL2 in aspen, respectively [31].

In most cases reported, downregulated CAD plants had slight or no changes in lignin content, but some of these plants had a higher in vitro cell-wall digestibility [32,33]. It should be noted here that measuring lignin contents is problematic enough in normal plants, but there is obviously no guarantee that quantifying polymers that may be structurally quite different (particularly as a result of incorporating novel monomers), by any analytical method, is reliable. A higher cell-wall digestibility was observed in bm1 maize plants compared to normal plants, but lower than in bm3 plants [3]. Chen et al. [25] recently observed a 20% lignin decrease in CAD down-regulated tall fescue plants, with an 8.3 percentage points increase of in vitro dry-matter digestibility. However, according to data found in literature, the maize bm1 mutant appeared to be less efficient in cattle feeding. When bm1 maize were fed to sheep, only a slightly higher cell-wall digestibility observed [34], or an intermediate value between normal and bm3 isogenic types (107, 100 and 112%, respectively) [35]. Similarly, the average daily gain weight of bulls fed a bm1 hybrid was 105% of the value observed in animals fed the isogenic normal hybrid, whereas it was 118% when bulls were fed the bm3 isogenic hybrid. Grabber et al. [36] demonstrated that, when the effect of lignin composi-

tion was isolated, hydroxycinnamaldehyde-containing lignins that may result from CAD down-regulation were less readily digested by crude polysaccharidases. The recent discovery of a SAD (sinapyl alcohol dehydrogenase) in aspen [37] questions the independence of pathways leading to guaiacyl and syringyl units in grasses. The FaCAD1b used in down-regulation of tall fescue plants had 97–99% identity in nucleotide and deduced amino acid sequences with the three other CADs found in the cDNA library [25]. But CAD and SAD from aspen had only 53% amino acid sequence identity [37], and *Arabidopsis* AtCAD-C and AtCAD-D shared only 75% amino acid identity, the later being more specifically involved in the synthesis of S units [38]. Both results strengthen the likelihood of another different CAD type in fescue and in maize [3]. The down-regulated tall fescue plants exhibited a 36 and 49% residual activity against coniferaldehyde and sinapaldehyde, respectively, but the activity against *p*-coumaraldehyde was not modified. Transgenic plants had correlatively a lower release of G and S units after thioacidolysis, but similar H-unit release. CAD was demonstrated to be a useful target for paper pulping quality improvement in dicotyledons [39–47] and gymnosperms [48,49]. CAD down-regulation should now be considered for forage digestibility improvement in monocotyledons, even if it is not the most promising target. The specificity towards G or S units synthesis following CAD down-regulation also remains to be assessed.

Similarly, before concluding as to the relevance of CCR engineering for grass improvement, it is necessary to further elucidate the possible specificity of different CCRs leading to guaiacyl and syringyl units. Severely CCR-downregulated plants have exhibited serious growth defects and may exhibit a wounding response [40,50–53]. Simultaneous down-regulation of two (or more) genes, however, may result in a general lignin down-regulation and produce plants without some of the deleterious features of down-regulating individual genes. Tobacco hybrids resulting from the crossing of transgenic lines down-regulated for CCR and CAD, CCR and COMT (caffeic acid *O*-methyl transferase), or COMT and CAD, had a reduced lignin contents, but with no adverse impact on the growth of the plants in greenhouse experiments [54–56]. Abbott et al. [54] demonstrated that chimeric silencing constructs could be more efficient than achieving multiple suppressions by crossing independent events. This strategy should allow a synergetic enzyme reduction that regulates the flux of metabolites through the lignin pathway. The interest in simultaneously down regulating CAD and CCR genes must be investigated in grasses.

The ability to measure S/G ratios is not complemented with an ability to measure the levels of incorporation of novel monomers into lignins in mutant or transgenic plants. Thus, although thioacidolysis marker compounds are available for CAD and COMT deficiencies, for example, the levels of incorporation of hydroxycinnamaldehydes or 5-hydroyxconiferyl alcohol monomers into the lignin cannot be so readily estimated. As an example, COMT-deficient poplars can liberate up to 12% levels of a 5-hydroxyguaiacyl monomer in thioacidolysis [57], yet NMR reveals that the 5-hydroxyguaiacyl contents are much higher, perhaps 25% of the β-ether level [58]. This is because thioacidolysis does not fully cleave the benzodioxane structures formed as a result of 5-hydroyxconiferyl alcohol incorporation [59]. Indeed dimeric benzodioxane units can be released by thioacidolysis or the DFRC method, but these do not quantify as highly as their guaiacyl or syringyl counterparts, simply because they are incompletely released and because chains of repeating benzodioxane units are formed in these lignins [60]. The point here is that explanations for different pulping or digestibility characteristics may have overemphasized the effect of the S/G ratio (because that is what has traditionally been measured), when in fact it is the structural alteration caused by the introduction of novel monomers into the lignification process that may have the major impact. For example, the reduced pulping performance of COMT-deficient poplars is less likely due to the reduced S/G than it is to the fact the syringyl β-ethers in the wild type poplars have been largely supplemented by benzodioxane structures in the COMT-deficient transgenics. Although benzodioxanes are also strictly β-ether units, they will not cleave under pulping conditions [61]. Similarly, an overlooked aspect of the incorporation of either hydroxycinnamaldehydes or 5-hydroxyconiferyl alcohol into lignins is that the intermediate quinone methides formed involving these structures rearomatise by internal reactions rather than by addition of external nucleophiles [58]. The quinone methides are therefore not available for attack by

polysaccharide OHs or uronic acids. Mechanisms for cross-linking polysaccharides to lignins are therefore lost in these units, which may well contribute to the digestibility improvements.

Down-regulation of 4CL, driven by a lignin tissue xylem-specific promoter, is a promising approach toward reducing lignin content, as illustrated by the work of Hu et al. [62] in aspen showing a reduction up to 45% in lignin content. Based on current knowledge and hypotheses on the lignin pathway [3], CCoAOMT (caffeoyl-CoA *O*-methyltransferase) is probably a major hub in controlling lignification, and probably also cross-linking in grasses because ferulic acid very likely originates from coniferaldehyde oxidation [63], and is therefore an ideal target for digestibility improvement. To date, only the work of Guo et al. [64] has illustrated the efficiency of CCoAOMT down-regulation on digestibility improvement, but in a dicotyledonous plant. Alfalfa plants with a 5% residual CCoAOMT activity had an increased cell-wall digestibility of 34%. No changes were observed in ADL content, whereas KL content was reduced by 25%. But once again the possible independence of CCoAOMTs involved in the G and S pathways, and their spatio-temporal behaviors, have to be elucidated, especially since a construct against one CCoAOMT could down-regulate two or more genes which differ only slightly. In poplar, CCoAOMT genes exhibited precise cell-specific expression patterns [65]. The effect on ferulate ester and ether cross-linking needs also to be investigated for such constructs in grasses.

An *Arabidopsis* mutant defective in C3H (*p*-coumarate 3-hydroxylase) had a strongly reduced lignin content. Besides, its lignin was entirely devoid of the normal S and G units and composed almost entirely of H units [66]. Schoch et al. [67] had previously established in *Arabidopsis* that 3-hydroxylation of *p*-coumarate derivatives occurred via 5-O-shikimate and 5-O-D-quinate esters. Moreover, Boerjan et al. [19] considered that this "C3H is very likely the sole 3-hydroxylase in the phenylpropanoid pathway", and that "none of the C3 and C5 substitutions of the aromatic ring take place at the cinnamic acid level in monolignol biosynthesis". According to these authors, C3H could be considered "as a major control point in the production of C3 and C5-substitued phenylpropanoids", and then could be considered also as a relevant target for grass digestibility improvement. However, both for C3H and CCoAOMT, null mutants or heavily down-regulated plants will very likely be too adversely affected. Only partially down-regulated plants, or less efficient natural alleles (due to a limited nucleotide polymorphism) will likely be efficacious in grass breeding.

The bm3 maize is thus far the most widely studied model available for improving digestibility and ingestibility through endogenous enzyme silencing. CaldOMT (5-hydroxyconiferaldehyde *O*-methyltransferase, or COMT), and Cald5H (coniferaldehyde 5-hydroxylase, or F5H ferulate 5-hydroxylase), could then also be considered as key targets in forage digestibility improvement. Among data published on COMT down-regulation in dicotyledons [17,19,23], the greatest improvement in cell-wall digestibility was observed in tobacco in which both a decrease in lignin content and an unexpected increase in S/G ratio were observed [68]. Piquemal et al. [23] recently reported the first COMT down-regulation in grasses (maize). In plants with 30% COMT residual activity, they observed a 9 percentage points increase in maize cell-wall digestibility. This increase was similar to those observed in the bm3 isogenic lines. Most recently, He et al. [24] obtained transgenic down-regulated COMT maize having an average 20% less lignin in stalks, and a correlated 7% improvement in stalk digestibility. The drawback of CaldOMT (and likely Cald5H) down-regulation or silencing was seen as the correlative S/G decrease, because a higher S/G ratio has been considered likely to positively impact the cell-wall digestibility in maize [69]. However, the case was not so clear cut for *Arabidopsis* F5H-up-regulated transgenics [70]. And, as noted above, S/G figures do not convey all of the structural changes, and the replacement of much of the S-unit content by 5OH-G units is likely to increase digestibility (as these units are not involved in cross-linking with cell-wall polysaccharides). Silencing or down-regulation of this gene achieved to date has led to a weaker agronomic value in some plants having a lignin reduction. Whether these traits can be overcome, or limited by lower down-regulation of the gene, is still questioned and being evaluated.

The optimal construct could be designed to reduce the expression of one gene while increasing the expression of another. The simultaneous down-regulation of CCoAOMT and over-expression of F5H

could be hypothesized as one pathway toward building a forage ideotype, assuming that this ideotype should have a reduced lignin content with an increased S/G ratio. This hypothesis is strengthened by recent results. Li et al. [71] demonstrated that aspen with a simultaneous down-regulation of 4CL and an overexpression of Cald5H had both a lower lignin content and a higher S/G ratio (and also more cellulose). Huntley et al. [72] upregulated F5H (Cald5H) with a C4H-promoter in poplar to achieve extremely high S/G ratios (∼13 : 1) and greatly increase pulping efficiency. Such S/G ratios, like those observed in F5H-upregulated *Arabidopsis* [58,73], are far higher than any observed in natural plants, where kenaf bast fibers are perhaps as high as any (∼7 : 1) [74]. This approach needs testing in grasses since F5H up-regulation in dicots can display disperse characteristics. For example, in *Arabidopsis*, F5H up-regulation is strikingly efficient in shunting monolignol production past the coniferaldehyde/coniferyl alcohol stage. The syringyl:guaiacyl ratio, estimated from NMR, is greater that 97 : 3 [58,73]. However, again to illustrate the problem with measuring only S/G ratios, F5H-upregulated *Arabidopsis* appears to produce the 5-hydroxy-substrate at a rate that cannot be accommodated by the following COMT. As a result, the lignins contain significant amounts of 5-hydroxyguaiacyl units (from 5-hydroxyconiferyl alcohol incorporation) [58]. In aspen, however, the COMT is far from limiting, and F5H up-regulation causes no identifiable increase in 5-hydroxyguaiacyl units, only an S/G increase [71]. Again, since 5-hydroxyconiferyl alcohol incorporation into lignins appears to improve digestibility, presumably by reducing lignin-polysaccharide cross-linking, F5H up-regulation in grasses needs to be examined to determine if it results in higher S/G only, or in 5-hydroxyconiferyl alcohol incorporation. In either case, but particularly the former, manipulating an untested gene/enzyme combination is an intriguing approach; i.e. up-regulating F5H while down-regulating COMT will enhance the incorporation of 5-hydroyxconiferyl alcohol by pushing the system past coniferyl alcohol but limiting its ability to produce the ultimate, fully methoxylated, sinapyl alcohol. The limit to which this incorporation of the 'rogue' 5-hydroxyconiferyl alcohol monomer can be tolerated by the plant is currently under examination in several labs.

The monolignols polymerisation reactions may also be considered as good targets, even though multi-gene families encode laccases and peroxidases. Comparison of antisense data with data obtained in knock-out mutants will be especially of interest for genes belonging to multi-gene families. Unpublished Génoplante data has shown that the genomic sequence of a peroxidase involved in the lignin pathway was sufficiently specific and allowed its amplification without ambiguity. Barcelo [75] postulated that peroxidases were the sole enzymes involved in the ultimate step of lignin biosynthesis, but most recent reports consider the involvement of both laccases and peroxidases in the formation of phenoxy radicals [16,76]. Mechanisms of phenoxy radical coupling of monolignols are now well understood, and the coupling and cross-coupling propensities of the various phenolics are being elucidated [19,77]. Recent challenges [78] to the theory of chemically controlled phenolic coupling, claiming that defined lignin polymers must arise through absolute structural control by the likes of dirigent proteins, are simply too unwieldy and can not accommodate what is well known about the structure of the racemic lignin polymer [19,79]. Lignin deposition in cell walls is regulated by both monolignol synthesis and the transport of their precursors out of the cytoplasm, but the process of lignification remains best described as a simple chemical process. Transport forms have not been clarified so far [80]. 4–*O*–β-D-Glucosides, presumably synthesized by UDPG-utilizing glucosyltransferases and subsequently hydrolysed by specific β-glucosides, are possible candidates [42], but have only been well documented in gymnosperms (pine). However, two UDPG-glucosyl transferases were identified in the *Arabidopsis* genome, similarly to an ortholog of pine coniferin-β-glucosidase [81]. But no evidence for the involvement of these enzymes in the lignin pathway has yet been obtained and the actual mechanism of monolignol transport to the cell wall remains unresolved. Genes involved in monolignol transport could be targets for plant engineering for cell-wall digestibility improvement, but only if feedback mechanisms prevent accumulation of toxic phenolics in cells.

Genes (and promoting regions) involved in lignification spatio-temporal regulation have to be considered as potential targets for cell-wall digestibility improvement in plants. Myb-related transcription

factors are involved in regulating phenylpropanoid metabolism, and lignin content was heavily reduced in mature parts of tobacco plants over-expressing an Antirrhinum Myb factor [82]. No measurements of digestibility were given in this paper. Because Myb genes in maize belong to a very large family of expressed regulatory proteins [83], identification of Myb factors specifically involved in the lignin pathway is necessary before using them for cell-wall digestibility improvement. Similarly, LIM proteins appear to be involved in regulating gene expression in the lignin pathway [84]. The knowledge of regulating the patterns of lignin deposition is probably a crucial point to take into account before any genetic engineering could be reliably considered. To date, data on cell-wall composition and digestibility in genetically modified plants (and in naturally occurring mutants) were thus often difficult to predict. More data will be also necessary concerning suitable promoters to drive each transgene expression in grasses.

5. Conclusion

An understanding of the molecular basis for cell-wall digestibility will be at the forefront of further progress in grass cell-wall digestibility. Breeding targets could be revealed from both QTL mapping and candidates gene colocalization, and from genetic engineering in the lignin pathway or in pathways involved in other aspects of cell-wall biosynthesis. Moreover, for such complex traits, and especially when including ingestibility, a breeding target must be clearly identified before efficient knowledge of genetic resources will become usable for breeding. It is indeed not possible to cross an elite line with a genetic resource of interest for feeding value, but of poor agronomic value, without knowing why this resource is of interest and what part of the genome has to be introgressed. A genetic resource can be considered of interest only because a genomic trait related to feeding has been proven and localized in it, thus allowing a targeted introgressing in the building of a new genitor. Similarly, the improvement of forage cell-wall digestibility through genetic engineering supposes an a priori knowledge of the role of targeted genes or regulating factors. However, the clue for genetic target validation is a comprehensive knowledge of the biochemical patterns of cell-wall biosynthesis. But reciprocally, a genetic target for manipulation can be identified by in-depth studies of the biochemical characteristics of the cell wall in plants up- or down-regulated for a gene for which the function not understood.

The understanding of grass lignification and phenolics, and correlatively grass cell-wall digestibility, can also be based on research developed on model plants. To date, no work appears to have been published on manipulation of the lignin pathway in rice, with the exception of PAL at the beginning of phenylpropanoid biosynthesis [85] and peroxidases that were probably involved in wounding rather than lignification [86], even though this species is the model monocotyledon whose complete genome is nearly fully sequenced and has a high level of synteny with other grasses [87]. The maize genome is not fully sequenced (and numerous data are not publicly available). The search for genes and regulation mechanisms involved in cell-wall biosynthesis has thus to be also based on data obtained from different model plants, including *Zinnia elegans* that is a model system of *in vitro* xylogenesis [88,89], followed by the search of maize orthologs in genomic libraries. The *Arabidopsis thaliana* genome was completed in 2000 and was found to code for around 25,500 genes [90]. Numerous papers are devoted to lignification studies in *Arabidopsis*, both in primary and secondary wall, and significant discoveries were recently based on studies in *Arabidopsis*. Costa et al. [91] proposed an in silico assessment of phenylpropanoid pathway gene function and organization in *Arabidopsis*. Loblolly pine [92], quaking aspen [93] and zinnia [94] have entered the stage of expression sequence tag (EST) sequencing. These data together represent an important amount of sequencing from which genes implicated in grass lignification can be evidenced. In order to assign a putative function to these new unknown grass genes, a general research of homology with already known genes may be used. In order to validate these hypothetical functions, different functional genomic approaches can be used (transgenesis, transposon tagging, etc.). Other approaches currently opening on regulating and co-regulating mechanisms are based on transcriptomics, proteomics, metabolomics and phenomics [95]. Nowadays, transcriptome analysis, such as creating micro- or macro-arrays specifically devoted to the maize lignin pathway, is the most used or

most suitable. This transcriptome analysis will provide a fingerprint of cell-wall metabolism in maize, a model for other grasses, and will reveal both new genes and co-regulating mechanisms involved in grass phenolics. Maize could be considered the leading grass (and monocotyledon) model for investigations on lignification and its relationships with cell-wall digestibility. Targets considered of interest in maize should very likely be extrapolated to other monocotyledon forages, including C_3 plants.

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