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# Genetic and molecular basis of grass cell-wall degradability. I. Lignin–cell wall matrix interactions ☆

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**Abstract**

Lignification limits grass cell-wall digestion by herbivores. Lignification is spatially and temporally regulated, and lignin characteristics differ between cell walls, plant tissues, and plant parts. Grass lignins are anchored within walls by ferulate and diferulate cross-links, *p*-coumarate cyclodimers, and possibly benzyl ester and ether cross-links. Cell-wall degradability is regulated by lignin concentration, cross-linking, and hydrophobicity but not directly by most variations in lignin composition or structure. Genetic manipulation of lignification can improve grass cell-wall degradability, but the degree of success will depend on genetic background, plant modification techniques employed, and analytical methods used to characterize cell walls. ***To cite this article: J.H. Grabber et al., C. R. Biologies 327 (2004).***


**Résumé**

**Bases génétiques et moléculaires de la biosynthèse et de la biodégradabilité des parois de graminées. I. Interactions au sein de la matrice lignines × composés pariétaux.** Les lignines sont des polymères phénoliques complexes, avec une forte régulation spatio-temporelle, qui limitent la dégradation des parois végétales par les herbivores. Les lignines de graminées ont de plus la spécificité de posséder des acides hydroxycinnamiques (acide *p*-coumarique, acide férulique et ses dimères) liés en ester ou en éther aux polymères pariétaux. La variabilité de dégradabilité des parois est liée à la variabilité de la teneur en lignines, au caractère plus ou moins linéaire ou réticulé du réseau lignine et à l'importance de la réticulation par l'acide férulique (ou de ses dimères). Le succès d'une modification par voie génétique de la lignification sera lié au fond génétique et à la stratégie de sélection utilisée, mais aussi aux méthodes analytiques employées pour caractériser les polymères pariétaux. ***Pour citer cet article : J.H. Grabber et al., C. R. Biologies 327 (2004).***


☆ This article as well as the following one is part of a group of three articles, the second one of which will be published in a forthcoming thematic issue, on *Lignified Cell Walls* dedicated to Dr Bernard Monties.

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## 1. Introduction

Grass (*Poaceae*)-dominated ecosystems comprise about one third of the Earth's vegetative cover [1]. Grasslands and savannas, covering about 20% of earth's landscape, are a major source of nutrients for wild and domesticated herbivores. Graminaceous cereals (maize, rice, wheat, oats, rye, etc.) dominate cultivated cropland, supplying most of the dietary energy needs of people and many classes of livestock. Considerable genetic variation for cell-wall digestibility in grasses has been established both between and within species. In maize for example, *in-vivo* cell-wall digestibility of early maturing hybrids varied from 35 to 60% [2]. Lignins are likely the only component in cell walls resistant to bacterial and fungal degradation in the gut and their association with other matrix components greatly influences cell-wall properties, including the enzymatic degradability of structural polysaccharides [3,4]. In recent years, research by Dr. Bernard Monties and others have greatly increased our understanding of lignin formation in plants and mechanisms by which lignification limits the digestion of cell walls.

Most studies related to the genetics, genomics, and biochemistry of lignin have been conducted with woody dicot or gymnosperm species, whereas most forage plants are grasses (except protein-rich forage plants such as alfalfa, clovers, or amaranths). This focus is due to the major interest in reducing the cost and environmental impact of removing lignin from woody plants during pulping for paper production. Biochemical traits involved in lignin biosynthesis and cell-wall degradability have, however, been investigated in many forage grasses. Among grasses, maize has probably received the most attention in genetic and genomic studies related to cell-wall lignification and degradability [2]. The objective of this paper is to review lignin chemistry and lignin-matrix interactions in cell walls and their influence on forage grass digestibility.

## 2. Lignins in grass cell walls

The first step of lignin biosynthesis in grasses is the deamination of l-phenylalanine or tyrosine by ammonia lyases, yielding cinnamic or *p*-coumaric acids, respectively. Successive steps of hydroxylation, methylation, formation of hydroxycinnamoyl-CoA thioesters, reduction of hydroxycinnamoyl-CoA thioesters to hydroxycinnamaldehydes, and reduction of hydroxycinnamaldehydes leads to three *p*-hydroxycinnamyl alcohols (monolignols), *p*-coumaryl, coniferyl, and sinapyl alcohols which are transported from the cytosol to the apoplast. A number of proposals regarding the preferred pathway of monolignol biosynthesis have been recently reviewed [5]. Once in the apoplast, monolignols undergo dehydrogenative polymerization via oxidases to form lignins comprised of two major unit types – guaiacyl (G), derived from coniferyl alcohol, and syringyl (S), derived from sinapyl alcohol. *p*-Hydroxyphenyl units (H), derived from *p*-coumaryl alcohol, occur as a minor component of lignin.

Although composed of only three building blocks, the composition and structure of lignins varies considerably within and among plants [6,7]. Various metabolic intermediates are also components of lignin, particularly in plants with perturbed monolignol biosynthesis, and these are described in parts 2 and 3 of this review [8,9]. Lignin units are interconnected primarily through labile $\beta$-*O*-4 and $\alpha$-*O*-4 ether bonds and smaller amounts of so-called "condensed" C-C ($\beta$–5, $\beta$–$\beta$, and 5–5) and biphenyl ether (4-*O*-5, and 5-*O*-4) bonds that are resistant to chemical degradation. Analysis of degradation products following cleavage of ether linkages by thioacidolysis indicates the respective proportions of H, G, and S units in lignins are 4, 35, and 61% for mature maize stalks, 5, 49, 46% for wheat straw, and 15, 45, 40% for rice straw [10]. In contrast to grasses, the lignins of dicot stems contain 14 to 66% G units, with the balance consisting of S units and only trace amounts of H units. In gymnosperms, G units comprise at least 95% the lignin with the balance consisting of H and

trace amounts of S units. Exceptions are unusual gymnosperms such as *Ephedra* and *Gnetum* where S units make up 31 and 51%, respectively, of the lignin units. In more primitive plants such as Pteridophyta, H, G and S units are observed in diverse proportions depending on the species [10]. The S/G ratio in forage lignins increases as the plant ages [11–13]. In a recent study [13], lignin content increased moderately during stem elongation of tall fescue, but a major increase occurred when plants changed from the elongation stage to the reproductive stage. Lignin content in the cell wall at anthesis was ten-fold higher than at the beginning of the elongation stage. Simultaneously, S lignin content and S/G ratio increased with progressive maturity of stems. There was also a major decline in ruminal degradability of stem tissues, highly related to the increase in lignin content.

According to the comprehensive studies of Terashima and co-workers [14], the incorporation of H, G, and S units in grass lignins is spatially and temporally regulated and it varies between primary and secondary cell walls and among tissues. The incorporation of H and G units takes place at the onset of lignification in cell corners and the middle lamellae. Syringyl lignins have however, been detected in immature maize coleoptiles, suggesting that their deposition begins at the early stages of lignification [15]. Subsequently, the deposition of G with increasing proportions of S units occurs during lignification of primary walls of parenchymatous tissues and primary and secondary walls of xylary tissues and sclerenchyma fibers. Normally lignification proceeds from primary to secondary cell walls in forage grasses and legumes [14,16,17] but the reverse was observed for sclerenchyma in alfalfa [18]. Xylary tissues and sclerenchyma are lignified before parenchyma and they have a higher S/G ratio and lignin content at maturity [19–21]. Compared to parenchyma and other tissues with more limited lignification, the cross-sectional proportion and cell-wall thickness of xylary and sclerenchyma tissues in leaves and stems also increases during plant maturation [13,22,23]. Therefore, the increase in S/G ratio during plant maturation and lignification is due to the pattern of monolignol deposition into cell walls and the accumulation highly lignified xylary and sclerenchyma cells which are highly enriched in S lignins. These changes in lignin composition are accompanied by changes in lignin structure. Guaiacyl-rich lignins in middle lamella/primary wall are thought to be highly branched due to rapid polymerization and coupling by mainly condensed bonds. In contrast, syringyl-rich lignins may be deposited more gradually in secondary walls and would be more linear due to extensive coupling by $\beta$-$O$-4 bonds [24–26]. Polymerization of monolignols in a cell-wall matrix enhances the formation of lignin and coupling by $\beta$-$O$-4 bonds [27–29]. Although lignin deposition in individual leaves or internodes takes place over a period of weeks [30,31], in individual cells this process is completed in a two to four day period in leaves [32,33], and a probably a longer period in internodes [34] but this has not been adequately characterized.

## 3. Hydroxycinnamates in grass cell walls

In grasses, as in other Commelinoid monocotyledons, hydroxycinnamic acids, namely *p*-coumaric and ferulic acid (along with its array of dehydrodimers), are ester and/or ether-linked to cell-wall polymers. Ester-linked caffeic acid is a minor component in some forage grasses [35]. Sinapic acid esters have recently been implicated in cell-wall cross-linking as sinapic acid, sinapate dehydrodimers, and even sinapate-ferulate cross-products were released by saponification from cereal grains, notably wild rice, rice, wheat and spelt [36]. Depending on the tissue and its stage of development, cell walls in C4 grasses tend to have higher levels of hydroxycinnamic acids than C3 grasses; maize and sorghum cell walls can contain up to 4% ferulates (monomers plus dimers) and up to 3% *p*-coumarate [21,37].

According to Chase et al. [38], cell-wall-bound ferulic acid is restricted to the terminal groups of Commelinoids, including Poaceae (grasses). In grasses, feruloylated arabinoxylans are formed intracellularly and then exported to the maturing wall [39]. Peroxidase-mediated dimerization of ferulate into diferulates probably occurs mainly in cell walls [40,41], although some dimerization may occur within Golgi vesicles prior to xylan deposition into the apoplastic space [42,43]. Over 50% of wall ferulates can undergo dehydrodimerization, forming a large array of 8-coupled diferulates and small amounts 5–5-coupled diferulate [40,41,44]. Diferulate cross-linking is commonly thought to play a role in stiffening cell walls and de-

celerating growth [33,45,46]. Ferulate monomers and dimers may also have an important role as initiation sites for lignin formation and for anchoring lignin in grass cell walls [47–49]. During lignification, ferulate and 5–5-coupled diferulate copolymerize more rapidly with monolignols than 8–5-, 8-*O*-4-, and 8–8-coupled diferulates but the final extent of incorporation of all isomers can exceeded 90% [41]. Ferulate monomers are linked to lignins by various types of ether [47,50, 51] and C-C bonds [48,49,52]. Hydrolysis of $\beta$-*O*-4 and $\alpha$-*O*-4 ether linkages releases about 35% of the ferulate monomers and 25 to 65% of the various diferulate isomers incorporated into lignin [41] but isomers linked to lignin by C-C, styryl ether and biphenyl ether bonds cannot be released by current solvolytic methods. Some of the C-C linked ferulates, for example the 8-$\beta$-cross-linked structures, can be observed by NMR in synthetic lignins [52,53] and in isolated grass lignins [48]. Recently, ferulate trimers (dehydrotriferulates) have also been identified following their alkaline release from bran cell walls. The first trimer isolated independently by two groups [54,55] was a 5–5/8-*O*-4-trimer. Other trimers are currently being isolated and identified. It is not yet known whether dehydrotriferulates represent cross-coupling of three independent arabinoxylan chains or just two; the latter is suspected, but verification awaits an insightful approach. The type of ether linkage formed between ferulates and lignins remains a point of controversy; most studies indicate that ferulate ethers derive from oxidative coupling to the $\beta$-position of lignin units [47,49, 52] while others support preferential ether bonding of ferulic units at the benzylic position ($\alpha$-ether linkage) of lignin units [56]. The latter case, concluding that ferulates are essentially all benzylic $\alpha$-ethers is unfortunately flawed by the use of reaction conditions that also cleave $\beta$-ethers (F. Lu and J. Ralph, unpublished). As a result of these coupling reactions, arabinoxylans become extensively cross-linked by ferulate dimerization and by incorporation of ferulate monomers and dimers into lignin. The concentration of alkali-labile ferulates initially increases during primary wall formation and then peaks and declines during secondary wall formation and lignification [12,57]. This reduction in *measurable* ferulate during later stages of cell-wall formation has been used to support the contention that ferulate deposition is limited to primary cell walls [58]. However, recent studies have shown that at least 50–70% of alkali-labile ferulate deposition occurs during secondary wall formation and lignification [31,33]. These analyses, however, again underestimate ferulate and diferulate deposition in secondary walls because radical coupling of ferulate and diferulates to lignin prevents the recovery of most of these acids by the solvolytic methods used to degrade lignin [41]. Immunocytochemical studies with maize stems also indicate that ferulates are deposited in lignified walls of secondarily thickened xylem, sclerenchyma, phloem fibers, and parenchyma tissues [59]. In mature sorghum, ester and ether-linked ferulate concentrations were greater in sclerenchyma and vascular tissues than in pith parenchyma and epidermal cells [21]. Grass lignins may also be bound directly to other wall polymers by benzyl ether or ester bonds resulting from opportunistic nucleophilic addition reactions between quinone methide intermediates of lignin and hydroxyl or acid groups on non-cellulosic polysaccharides and proteins [60–62]. While it is well established that cellulose and hemicellulose components are tightly associated through hydrogen bonds, the occurrence of covalent bonds between cellulose and other cell-wall constituents has, thus far, not been demonstrated.

*p*-Coumarate is mainly esterified to the $\gamma$-position of phenylpropanoid sidechains of S units in lignin [63–65]. Although very small quantities of *p*-coumarate are esterified to arabinoxylans in immature tissues, most *p*-coumarate accretion occurs in tandem with lignification [12,66], making *p*-coumarate accumulation a convenient indicator of lignin deposition. Structural studies suggest that syringyl units are enzymatically pre-acylated with *p*-coumaric acid prior to their incorporation into lignin, so sinapyl *p*-coumarate is the logical precursor incorporated into lignin [65]; analogous preacylation of sinapyl alcohol by acetate has been recently established in kenaf [67]. The role of *p*-coumarate units is not understood. In vitro studies indicate that *p*-coumarate esters enhance the oxidation of sinapyl alcohol [68–71]. In contrast, the artificial polymerization of syringyl-rich lignins into primary maize walls was at times depressed by sinapyl *p*-coumarate, due to accelerated inactivation of peroxidase and disruption of ferulate-lignin cross-linking (J.H. Grabber, unpublished). As noted above, ferulates probably act as nucleation sites for lignin formation and their extensive copolymerization with monolignols helps to anchor lignins into cell walls during the

early stages of lignification. Once most ferulates become incorporated into lignin, sinapyl *p*-coumarates may aid the formation of syringyl-rich lignins during the latter stages of lignification. Despite their phenolic nature *p*-coumarate esters on lignin units form few, if any, cross-linked structures mediated by radical coupling reactions of the *p*-coumarate moiety [63]. This appears to be because cross-coupling reactions are inefficient, and because radical transfer to monolignols and free phenolic lignin units is rapid. For example, in reactions of *p*-coumarate esters and sinapyl alcohol, the *p*-coumarate remains unreacted until all of the sinapyl alcohol is depleted [71]. Because *p*-coumarates remain as free-phenolic pendant groups on lignins, room-temperature alkaline hydrolysis provides a good estimate of the total quantity of *p*-coumarate in cell walls. *p*-Coumarate can, however, undergo a photocatalyzed cyclodimerization during tissue development to form ester-linked cyclobutane derivatives, the truxillic and truxinic acids [72–74]. Since virtually all *p*-coumarates are esterified to lignin, their cyclodimerization probably results in cross-linking of lignin polymers. Cyclodimers between *p*-coumarate and ferulate are also formed in grasses and these may cross-link lignins to xylans. Cyclodimers involving two ferulate moieties are comparatively rare. In mature sorghum, *p*-coumarate concentrations varied considerably between tissues, with extremely low levels in epidermis, moderate levels in sclerenchyma and high concentrations in vascular tissues and particularly pith parenchyma [21].

For nearly a century, it has been known that a substantial fraction of grass lignins (25 to 50%) can be solubilized by alkali at room temperature [75]. This is the reason why alkali treatment of small grain cereal straws improves their organic matter digestibility by more than 25% [76]. The alkaline solubility of grass lignins, which is not observed with herbaceous dicot and wood lignins, may be accounted for by two structural properties of these polymers. The first is the occurrence of the aforementioned ferulate cross-links between cell-wall polymers. The alkaline hydrolysis of ferulate esters would decrease the cross-linking between arabinoxylans and lignin thereby enhancing the enzymatic degradation of polysaccharides. The second and more likely reason is the higher frequency of free phenolic groups in grass lignins, as compared to lignins in other types of plants. When ionized, these abundant phenolic groups would be mainly responsible for the solubilization of grass lignins in alkali. Such a hypothesis is supported by the fact that methylation of phenolic groups with diazomethane reduces the alkaline solubility of grass lignins to the low level observed for other types of plants [77]. This loss in alkaline solubility occurs even though methylation has no effect on the alkaline hydrolysis of ferulate esters. Durot et al. [78] also established that the release of monomers by thioacidolysis was increased up to 137% after a NaOH treatment of wheat straw. This response to NaOH treatment might in part be due to the removal of *p*-coumarate esters from lignin, which reduce S units yields by thioacidolysis [64].

## 4. Genetic variation for lignin traits and cell-wall digestibility

Variation in lignin characteristics and digestibility was first observed in maize brown-midrib mutants in the 1960s and 1970s [79–82]. Since then, brown-midrib mutants of maize and other C4 grasses have been the subject of numerous studies and these are described in part 2 of this review [8]. Normal maize lines and hybrids also display substantial genetic variability for lignin and degradability traits, at times rivaling that observed with brown midrib mutants [83–90]. In these and other studies with grasses [91], degradability was negatively associated to lignin concentration, but this is not always the case [92]. Relationships between lignin content and degradability are greatly influenced by the hydrophobicity of lignin, its cross-linking to other wall components, the polymerization conditions in the apoplast, and probably other chemical or structural factors [62,93,94]. Methods used estimate lignin concentrations and cell-wall degradability also influence the relationship between these traits [2,95,96]. The anatomical configuration and cell-wall chemistry of various tissues is also a major determinant of cell-wall degradability in grasses [22,97–101]. Therefore, genetic engineering or plant breeding efforts aimed at reducing lignin content or other lignin traits can improve grass cell-wall degradability, but the degree of success may vary depending on genetic background and the plant modification and analytical methods employed [2]. In some cases, the degradability of maize

or other grasses was associated with the ratio of S/G units in lignin [11,87,102,103]. This has led some to suggest that cell-wall degradability is related to the relative ease with which lignin undergoes depolymerization during acid or alkaline hydrolysis of $\beta$-ether linkages. Although lignin depolymerization during pulping is positively related to the S/G ratio of lignin [104] the mechanisms controlling cell-wall digestibility are completely different, so the analogy is weak at best. According to another model [58], degradability of cell walls is influenced by the composition and three-dimensional structure of lignin, such that condensed coupling of H and G units into branched polymers limits enzymatic hydrolysis of structural polysaccharides to a greater degree than $\beta$-$O$-4 coupling of S units into linear polymers. Model studies with artificially lignified maize walls [62,105], however, suggest that varying ratios of H, G, and S units and branching of lignin (as indicated by the frequency of $\beta$-$O$-4 coupling) does not influence cell-wall degradability. Although variations in the H, G, or S unit composition of lignin or the frequency of $\beta$-$O$-4 coupling may not directly influence cell-wall degradability per se, they may at times be associated with lignin characteristics (e.g., lignin concentration and cross-linking) that do control cell-wall degradability. For example, reduced deposition of S lignins, through plant selection or direct gene manipulation, will enhance degradability if lignin concentration or distribution is reduced in cell walls. At times, however, plants will compensate for reduced S deposition by increasing G deposition, leading to no change in overall lignin content or cell-wall degradability [106]. This highlights the potential shortcoming of selecting or manipulating a trait that does not mechanistically control some aspect of cell-wall degradability.

Substantial variation for $p$-hydroxycinnamates (content and bonding modes) has been observed between normal maize genotypes hybrids or lines [86–88,107]. In these and other studies with grasses [92,108], the concentration of $\beta$-$O$-4 and $\alpha$-$O$-4 etherified ferulate was negatively correlated with cell-wall digestibility. The importance of ferulate and diferulate cross-linking in limiting the rate and, to a lesser degree, extent of cell wall degradation has been confirmed in model studies with non-lignified and artificially lignified walls of maize [94,109]. Progress in developing plants with lower levels of cross-linking is currently hindered by limitations in measuring all ferulate and diferulate cross-links by chemical or spectroscopic methods. Due to the limitations and complexity of current methods, typically only etherified ferulates are quantified by solvolytic analyses. Such analyses may account for as little as 15% of total cross-linking because C-C, 8-$O$-4 styryl ether, and biphenyl ether coupling of ferulate and diferulates to lignin are not determined [41]. The use of etherified ferulate to represent total cross-linking is even more tenuous because the proportions of $\beta$-ether coupled ferulate and diferulates are influenced by the pH and rate of lignin polymerization and possibly by the monolignol composition of lignin (J.H. Grabber, unpublished). Consequently, it is not known how well etherified ferulate reflects total ferulate and diferulate cross-linking in cell walls, particularly in cases where plant selection or gene manipulation alter the dynamics of lignin formation or its compositional makeup. Despite these limitations, direct plant selection for low levels of ferulate ether-linked to lignin has improved bromegrass cell-wall fermentation by rumen microorganisms [108]. Plant selection for this important trait would, however, be greatly enhanced if total ferulate and diferulate cross-linking in cell walls could be accurately and rapidly measured by solvolytic or spectroscopic methods.

The concentration of $p$-coumarate and the ratio of $p$-coumarate to ferulate released by saponification are often negatively associated with cell-wall digestibility [99,107,110,111]. Since most $p$-coumarate esters on lignin are not covalently attached to other cell-wall polymers, they should not directly influence cell-wall degradability [58]. However, cell-wall model studies indicate that $p$-coumarate interferes with ferulate-lignin cross-linking and in some cases reduces the proportion of artificial lignins bound to maize cell walls (J.H. Grabber, unpublished results). Ongoing work will determine whether $p$-coumarate directly (e.g., through cyclodimerization) or indirectly (e.g., through reductions in ferulate–lignin cross-linking) influences the degradability of cell walls. The ratio of $p$-coumarate to ferulate released by saponification may also have some utility as a selection criterion, with $p$-coumarate being a indicator of total lignin deposition and ferulate being an indicator of lignin distribution among plant tissues. As noted above, the esterification of $p$-coumarate to syringyl units in grasses makes

it a good index of overall lignification. Ferulate may be a good indicator of lignin distribution because it is deposited throughout primary and secondary wall formation in grass tissues and its release by saponification declines dramatically as cell walls undergo lignification. Therefore, at a given lignin concentration, a low *p*-coumarate to ferulate ratio might indicate that lignin deposition is restricted to a relatively low proportion of tissues, whereas a high ratio would suggest that lignin is more evenly distributed among tissues. This ratio may also indicate differences in the proportions of non-lignified and lignified tissues in grasses. Variations in the proportions, lignin distribution, and degradability of tissue types in grasses have been observed [22,97] but their relationship to the ratio of *p*-coumarate to ferulate needs to be more thoroughly examined before this compositional trait is used as a selection criterion in plant breeding programs.

Recent studies with a cell-wall model system indicate that benzyl ether and ester cross-links, formed by the nucleophilic addition of neutral and acidic polysaccharides to lignin quinone-methide intermediates, restricts the degradability of cell walls [62] (J.H. Grabber, unpublished results). Genetic variation for benzyl ester and ether cross-linking in grasses has not been investigated, largely because the abundance of these cross-links is not known and there is currently no method for directly measuring them on a routine basis.

## 5. Conclusion

Understanding of the molecular basis of cell-wall digestibility provides a foundation for improving grass cell-wall digestibility through manipulating the lignin pathway or other aspects of cell-wall biosynthesis. Grasses exhibit significant genetic variation in the degradability, composition and structure of cell walls and in the anatomical configuration and degradability of their tissues. But relationships between cell-wall chemistry, anatomy and cell-wall degradability are not fully established. Lignin content is often a primary determinant of wall digestibility, but breeding for a low lignin content can impair plant vigor. Cross-linking of lignin to structural polysaccharides, particularly via ferulates and diferulates, and lignin hydrophobicity are also major determinants of cell-wall degradability. A great obstacle for plant improvement programs is that many chemical and anatomical factors influence cell-wall digestion. So variations in one factor (e.g., cross-linking) may work with or against variations in other factors (e.g., lignin content) that determine digestibility. Perturbing one factor, by plant selection or transgenic methods may lead to compensatory changes in other factors that control digestibility. Plants may, for example, respond to lower lignification by increasing the amount of cell wall cross-linking [112], perhaps yielding no net change in digestibility. Our challenge is to accurately describe what factors control digestion and to develop an array of effective tools for genetic modification and selection of plants with improved digestibility.

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