The Impact of Cell Wall Feruloylation on Plant Growth, Responses to Environmental Stress, Plant Pathogens and Cell Wall Degradability

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Abstract: This article summarizes evolving concepts and scientific findings on cell wall feruloylation and ferulate oxidative coupling processes in grasses, and the effects these have on the wide range of cell wall properties and consequent plant responses to biotic and abiotic stress and tissue degradability. Updates of the different strategies that have been applied to genetically modifying cell wall feruloylation are presented. Special emphasis is given to the modification of cell wall feruloylation by heterologous expression of cell wall ferulic acid esterase, as this strategy has provided insights into the impact of feruloylation on the changes in the physicochemical properties of the cell wall with consequent effects on different plant processes. Emerging feruloyl transferase candidate genes codifying enzymes accounting for ferulate incorporation into grass arabinoxylans are also highlighted.

Keywords: ferulic acid; feruloylation; feruloyl transferase; cell wall; biofuels; digestibility; pathogens; grasses

1. Introduction

Ferulic acid is a phenolic compound that is anchored to terminal arabinofuranose residues of arabinoxylan in the primary and secondary walls of monocotyledonous plants via ester linkages [1–3]. Ferulates have also been found anchored to arabinan and galactan structural units of pectin in dicotyledonous plants [4,5]. These wall-bound ferulates are important components in plant cell walls, especially in grasses, as the oxidative action of cell wall peroxidase/H2O2 triggers ferulate coupling and the cross-linking of previously separated polysaccharide chains [6,7], resulting in the linkage of lignin to the xylan/cellulose network [8,9]. Such cross-linking affects a wide range of cell wall properties, such as resistance to enzymatic attack, with consequences for forage digestibility and biomass conversion to ethanol.

This article focuses on the impact that cell wall feruloylation and ferulate oxidative coupling processes have on the wide range of cell wall properties and the different approaches that have been used to elucidate these findings, with special focus on grass cell walls. Scientific evidence shows that arabinoxylan feruloylation has significant impacts on cell-to-cell adhesion, wall assembly, internode elongation, cell wall strength, plant resistance to pathogens and herbivores and cell wall degradability. Equally important to these findings has been the identification and testing of the candidate genes codifying enzymes accounting for hydroxycinnamic acids incorporation into grass arabinoxylans (AX), which has been challenging.

Grasses play a significant role in agriculture as feed for grazing animals (fresh forage) or for silage and hay (preserved forage). In addition, grasses also constitute a renewable alternative to produce biofuels. Understanding the process of cell wall feruloylation and the identification of the genes involved can contribute to a more efficient utilization of plant cell walls in the agriculture, food and bio-ethanol industries.
2. The Importance of Cell Walls to Plants

The plant cell wall, which is located at the surface of nearly every cell in the plant, comprises a dynamic and complex group of components, including cellulose microfibrils, several matrix polysaccharides (pectin and hemicellulose), proteins (including different structural proteins and enzymes) and phenolic compounds.

Plant cell walls fulfill a very wide number of functions far beyond merely mechanical support. The structural polysaccharide components of the cell wall determine cell size and shape and ultimately plant morphology (as reviewed by Tucker et al., 2018) [10], while providing mechanical strength, which gives cells the ability to resist turgor pressure, the cell expansion-driving force [11,12]. The process of cell growth, which is driven by turgor pressure, is only achieved due to the ability of the cell wall to modulate its mechanical properties by a process that involves loosening and tightening of its primary components. This in turn occurs through the regulation of its polysaccharide composition, their integration into the existing cell wall and their cross-linking within the wall [12].

Ferulate oxidative coupling of hemicellulose is an especially important type of cross-linking occurring particularly in grass cell walls and serves distinct roles in the life of plants. This will be discussed in more detail in a later section; however, it is worth mentioning that the rigid gel formed by cross-linked pectin [13] and possibly the xylan backbone of arabinoxylan and arabinose of rhamnogalacturonan (RG-I) by ferulates [14] mediates cell-to-cell adhesion, preventing cell migration.

Beyond its structural function, the cell wall also works as a versatile monitoring system whose physicochemical properties alter when faced with abiotic and biotic stressors, providing a key component of plant defense and adaptation systems [15–17]. This is the first barrier encountered by invading pathogens, and cell wall composition and re-modeling and the extent of cell wall cross-linking are orchestrated by over a thousand genes [10,18].

3. Cell Wall Structure

Consistent with the role it plays in different processes, plant cell wall structure shows great variation, not only between genera and species, but also between types of tissues. Plant cell walls are classified into primary and secondary. The non-lignified primary walls are laid down around young cells, allowing these cells to expand and provide both flexibility and structural support [19]. Once the cell stops dividing and growing, a thicker secondary cell wall is deposited in between the plasma membrane and the primary wall, providing strength and allowing upright growth [20].

3.1. Cellulose

Cellulose is comprised exclusively of unbranched β-(1,4)-linked D-glucose, where alternate glucose residues are rotated 180°, making cellobiose dimer as the repeating unit. An important result of this rotation is the linear conformation that the polymer undertakes, allowing for hydrogen bonding within chains and tight packing of glucan chains, making cellulose insoluble in water [21]. Glucan chains come together in a parallel packed array of 16 to 36 (or even more) chains to form a microfibril with an exceptionally high degree of polymerization (DP). The mean DP reaches 6000 in primary and 14,000 in secondary corn stover walls [22]. Cellulose biosynthesis takes place at the plasma membrane by a cellulose synthase protein complex (CSCs), building β-glucan chains from cytosolic UDP-glucose as the substrate [23,24].

3.2. Hemicellulose

In contrast to cellulose, hemicellulose polysaccharides are shorter than those of cellulose and quite often branched, with chains containing other sugars, phenolic and acetyl groups. Hemicellulose composition varies both with plant species and tissue type, and can be classified into distinct groups based on their polysaccharide composition. These include xylans, xyloglucans, mannans and mixed-linkage glucans [25]. A common feature among xylans is a backbone β-(1,4)-linked xylose. This can be substituted with arabinose and
glucuronic acid, which are often known as glucuroarabinoxylan (GAX) and constitute the major hemicellulose in the primary walls of grasses and related monocot species, including bioenergy crops, such as switchgrass, where they constitute up to 50% of the dry weight of some tissues in these species [25,26]. A xyloglucan backbone consists of β-(1,4)-linked glucose residues substituted with xylose. Xylosyl residues can be further substituted with galactose or arabinose, but other variations exist [26]. Mannans, which are β-(1,4)-linked polysaccharides containing mannose, and are often acetylated and mixed-linkage glucans consisting of β-(1,4)-linked glucans containing single β-(1,3)-linkages, are common in grasses [26]. Hemicelluloses can bind to cellulose surfaces, forming a matrix between cellulose fibers, playing a significant role in stopping fibers from aggregating [27]. Most of the hemicellulose polysaccharides are synthesized in the Golgi membranes by glycosyltransferases (GTs), using a variety of nucleotide sugars as substrate [28]. By interacting with cellulose and lignin, hemicellulose regulates cell expansion and adds strength to the wall [29].

3.3. Pectin

Pectin polysaccharides are a complex heterogeneous group of galacturonic acid-containing polysaccharides that are abundant in dicots and gymnosperms but largely absent in grass cell walls [30], and are synthesized in the Golgi apparatus by membrane-bound GTs.

They have a key role regarding cell adhesion in the middle lamella, holding the cell wall together [31]. Pectic polysaccharides comprise five structural classes of polymers: homogalacturonans (HG), rhamnogalacturonans I (RG-I), rhamnogalacturonans II (RG-II), xylogalacturonans (XGA) and apio galacturonans (AGA) [31]. HG, the most abundant of the pectic polysaccharides, are polymers of unbranched α-(1,4)-galacturonic acid chains [31,32]. A significant feature of the HG galacturonic acid residues is the methyl esterification of a proportion of the C-6 carboxyl groups following its biosynthesis, which blocks the acidic carboxyl groups [32]. A pectin methyl esterase (PME) enzyme hydrolyzes the methyl ester groups, unmasking the negatively charged carboxyl groups and restoring the ability of GalA residues to ionically interact with Ca$^{2+}$ and cross-link HG chains forming a rigid gel [33].

3.4. Lignin

Lignin is an irregular three-dimensional and heterogeneous phenolic polymer formed from the enzyme-mediated, free radical coupling of phenylpropanoid building blocks. These monolignols, p-coumaroyl, coniferyl and sinapyl alcohol, give rise to p-hydroxyphenyl (H), guaiacyl (G) and syringyl (S) subunits of lignin polymers, respectively. The monolignols are polymerized directly in the cell wall by peroxidase and laccase-assisted radical coupling [34]. Lignin composition is determined by the proportions of the different monolignols and the variety of covalent linkages formed between them [35], and is usually described by the abundance and ratio of these subunits [36]. Lignin from grasses is composed of S- and G-units with much less abundance of H-units [37]. An interesting feature of grasses is the presence of p-coumaric acid esterified to lignin residues at the γ-position, where in maize and bamboo, p-coumarates are preferentially acylated to S-units [38].

Lignin synthesis is mainly restricted to thickened cell walls of tissues involved in mechanical support (sclerenchyma and xylem fibers) and water transport (xylem vessels and tracheids). The presence of lignin in plants can be visualized by UV and Raman micro spectroscopy, histochemical Mäule staining (specific to S-units) and phloroglucinol-HCl staining (which reacts with lignin cinnamaldehyde end-groups). Phloroglucinol-HCl staining generates a fuchsia or pink color in tissues where these aldehydes are located, such as in the xylem and sclerenchyma fibers [39] (Figure 1). As shown, a pink/fuchsia coloration is observed in xylem and sclerenchyma fibers of two Brachypodium distachyon inbred accession lines 227011 (CWC) and 255730 (CWF) stem cross-sections, but is absent in cortex and pith tissues. Interestingly, coloration is absent in the epidermis of Bd line CWC
but is present in the CWF line. The presence of lignin in plant cell walls provides strength and increased mechanical support in stems, and creates waterproofing resistance, enabling long distance water transport in vascular tissues [34,40]. In addition, lignin also works as a physical barrier against pathogen attack [41], and may be induced upon pathogen infection [42]. Not surprisingly, its presence significantly reduces cell wall degradability by herbivores [43].

Figure 1. *Brachypodium distachyon* stem internode anatomy. Stem cross-sections 30 µm thick (between 3rd and 4th nodes, 7–10 days after the start of heading) from inbred accession lines 227011 (CWC) (a,d) and 255730 (CWF) (b,c), stained with phloroglucinol-HCl. The positions of the epidermis (Ep), sclerenchyma fiber (Sf), cortex (Co), vascular bundles (Vb), parenchyma (Pa) and pith (Pi) are indicated. Bar = 250 µm (a), 200 µm (b), 500 µm (c,d). Unpublished original data. Source M. Buanafina.

4. Plant Cell Walls as Food, Feed and a Source of Second-Generation Biofuels

Plant cell walls are an essential part of human life, as a source of dietary fiber and as fibers for clothing, paper, heating and shelter. Moreover, grass cell wall polysaccharides constitute the major source of carbon and energy for ruminants and serve as a renewable source of energy for biofuels to replace petroleum-based fuels for transportation needs.

Moore and Hatfield (1994) [44] defined dietary fiber as plant-derived food components that are not amenable to mammalian enzymatic hydrolysis. Although dietary fibers are considered indigestible in the human intestine, reaching the colon intact, they serve several important roles in digestive health. They consist mainly of cell wall components that can be subdivided into soluble (e.g., β-glucan, pectin, etc.) and insoluble (e.g., cellulose, some types of hemicellulose and lignin) materials, and can be obtained from legumes, cereals, fruits, and vegetables. Insoluble fibers are insoluble in water and gastrointestinal (GI) fluids and resist fermentation by the gut bacteria, presenting as dense particles that play a key role in bowel scouring [45]. Soluble fibers, in contrast, form viscous gels due to their ability to absorb water, and can increase the viscosity of digested food, delaying gastric emptying [46]. Soluble fibers are fermented by the gut microbiota and can impact interactions with bile acids, playing a significant role in lowering blood cholesterol levels, and as such, reducing the risk of cardiovascular diseases [47]. The overall organization and
physicochemical properties of dietary fiber constituents also affect nutrient bioaccessibility and digestion [48,49].

In contrast, ruminant animals have developed a sophisticated microbial ecosystem of anaerobic bacteria, protozoa and fungi [50,51], which secrete a diverse set of enzymes required for the hydrolysis of cell wall polymers [52], enabling them to utilize complex and recalcitrant cell polysaccharides, such as cellulose and hemicellulose [53]. Given that cattle can be raised on low-forage diets, several reasons can be laid down to support the use of forage at elevated levels in ruminant diets. When considering production costs, these are generally lower for forages compared to grains. In addition, perennial forages are considered more environmentally friendly since they offer protection against soil erosion and require reduced levels of pesticides and fertilizer, contributing to lower water pollution. Although forages stand as the primary nutritional source for ruminants, it has been stressed that these foods alone cannot meet ruminants’ energy requirements [54] due to the recalcitrant structure of the cell walls, which makes them of variable and in general low quality in terms of digestibility for ruminants. Cell walls also negatively affect intake by contributing to ruminant fill.

Since plant cell walls provide an abundant natural source of stored solar energy in the form of carbon, and comprise an assembly of biopolymers, the lignocellulose biomass (cellulose, hemicellulose and lignin), constitutes a renewable alternative for producing biofuels. First-generation biofuels, mainly ethanol from sugarcane, corn starch and oils (from food crops), have faced opposition due to claimed competition for land use and food production. Instead, the conversion to ethanol of plant cell walls derived from agricultural waste or dedicated energy crops constitutes the second-generation form of bioethanol, and represents a viable alternative to the use of food crops.

Economic conversion of cell wall lignocellulose to ethanol, which may seem a straightforward process, is not yet optimal, despite technical advances [55], and suffers from similar constraints to those faced by ruminants. One of the major limitations to the economic feasibility of this process lies in the recalcitrance of the cell wall against degradation into simple sugars (the saccharification process) [56,57]. The way these polymers aggregate and cross-link within the wall significantly influences the recalcitrance of biomass to enzymatic degradation, increasing the energy necessary for the lignocellulose biomass deconstruction for fermentation and conversion.

The current process of plant cell wall conversion to biofuel consists of physicochemical pretreatment at elevated temperatures or treatments with acids or alkalis, which aim to expose chemical bonds to a mixture of exogenously applied cell-wall-degrading enzymes to mediate their hydrolysis, and the subsequent conversion of polysaccharides to monosaccharides, followed by sugar fermentation to produce a variety of potential biofuels or chemical feedstocks. To make this process economically feasible, lignocellulose recalcitrance must be overcome and a commercially consolidated bioprocessing method developed, where cellulose and hemicellulose hydrolysis and hexose and pentose fermentation can occur concomitantly, using a single organism that can carry out both processes (the consolidated bioprocessing) (CBP); development efforts towards this end have been recorded [58,59].

5. Cell Wall Feruloylation and Cross-Linking in Relation to Lignification

As discussed earlier, the most abundant sugar side chains decorating the xylan backbone in grasses are α-arabinoses 1 to 3 linked to backbone xylopyranose (AX). An interesting feature of AX in the Poaceaes (grasses) is the presence of ferulic acid and p-coumaric acid [2,3]. Feruloylation refers to the process by which ferulic acid is anchored to the cell wall. In grasses, ferulic acid is attached to the cell wall by ester linkages at the arabinose O-5 position of the arabinoxylan side chain.

Feruloylation is an important feature, as these wall-bound ferulates provide the opportunity for cross-linking previously separated AX chains though ferulic acid oxidative coupling reactions mediated by peroxidases and hydrogen peroxide [6,7] and through photoisomerization by UV light [60]. Ferulic acid can also be ether-linked to lignin (via its
hydroxyl groups covalently attached to lignin monomers and oligomers), anchoring lignin in the cell wall and resulting in the linkage of lignin to the xylan/cellulose network via polysaccharide-ferulate-lignin complexes [8,9] (see Figure 2).

Figure 2. Schematic structure of ferulic acid (FA) cross-linking arabinoxylan (AX) and lignin in grass cell walls. The main β-(1,4)-linked xylan (Xyl) backbone is illustrated by dotted lines, the arabinose (Ara) side sugars are shown in circles and lignin is shown as gray-filled circles. Ester and ether bonds are shown by arrows as follows: 1. FA ester linked to AX. 2. 5-5' ester linked to FA dimer cross-linking AX chains. 3. FA ether linked to lignin. 4. AX ester linked to lignin. 5. Xylan ester linked to lignin. 6. Acetyl group ester linked to xylan. Modified from Williamson et al. (1998) [9]. Unpublished original data. Source P. Morris & M. Buanafina.

A number of ferulic acid dimers have been identified and characterized where the major forms are 5-5'-DFA, 8-O-4'-DFA, 8-5'-DFA (benzofuran cyclic form) and 8-8'-DFA (Figure 3). Ferulate dimers and tetramers have been identified in several plant species. Some examples include maize [61–64], tall fescue [65,66], Italian ryegrass [67] and wheat [68], and cell wall phenolics can be readily visualized by the autofluorescence of thin sections under UV light at 365 nm, both in vegetative and floral stems and in leaves of grasses such as Festuca (Figure 4).
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Currently there are two proposed sites for the cellular location of ferulic acid oxidative coupling and the consequent cross-linking polysaccharides to which they are attached. One is believed to take place in the cell wall and is supported by several studies. For
example, Grabber’s group showed that the level of cell wall ferulate monomers decreased, and ferulate dimer levels increased upon increased activity of wall-bound peroxidases and generation of H$_2$O$_2$ via glucose oxidase [69]. Likewise, Fry’s group, making use of cell suspension cultures fed with [$^{14}$C] cinnamate followed by monitoring of the kinetics of sugar binding and dimerization of [$^{14}$C]-labeled ferulate [63,70,71] and radiolabeled feruloyl-arabinoxylan trisaccharide (FAXX) [72], demonstrated that the formation of ferulate dimers, trimers and even higher molecular oligoferulates takes place in muro, and is depended on apoplastic H$_2$O$_2$ and wall-localized peroxidase. A second site for ferulate oxidative coupling may also be intracellular, as Obel et al. (2003) [73] have suggested, but restricted to 8,5′-DFA only, whereas the other dimers are formed in the cell wall. A more recent study [71] also supports intraprotoplasmic ferulate dimer formation, and contradicts Obel’s suggestion that only one dimer type is formed intracellularly by providing evidence that several dehydrodimers are formed intraprotoplasmically.

As mentioned earlier, ferulates have also been identified as initiation sites for lignin deposition in grasses [74]. Using maize cell suspensions that were synthetically lignified with coniferyl alcohol, Grabber et al. (2000; 2002) [75,76] characterized the cross-linked products formed between ferulates and coniferyl alcohol, and showed that during cell wall lignification, ferulates and diferulates became incorporated into lignin via 4-O-β′ and 4-O-α′ linkages, exceeding 90% of the total number of ferulates. Based on the kinetics of ferulate and diferulate incorporation, they were able to establish that ferulates and 5-5′-DFA specifically showed higher levels of copolymerization with coniferyl alcohol compared to 8-coupled diferulates, giving evidence for ferulates acting as nucleation sites for lignin polymerization.

6. Strategies for Manipulation of Feruloylation

We describe below three different techniques that can be used to genetically modify feruloylation of grass cell walls. The first technique is conventional plant breeding, including the use of existing mutants and forward and reverse genetics to obtain novel mutants; the second is genetic modification of structural or regulatory gene expression; and the third consists of the expression of transgenes that directly or indirectly reduce feruloylation either pre- or post-harvest. All three techniques have been assessed in both monocot and dicot species by several research groups, with varying degrees of success in modifying feruloylation of cell walls of different plant tissues. The main problems are pleotropic effects on plant growth and development and disturbed plant pathogen responses.

6.1. Conventional Plant Breeding

Plant breeding is based on the selection of recombinants derived from parents with largely non-overlapping desirable traits, and several strategies can be used to achieve this, including recurrent divergent selection, association and QTL mapping and the use of naturally occurring or induced mutants.

6.1.1. Divergent Selection

Examples of divergent selection for the levels of ester-linked diferulates of grass cell walls include maize populations selected for higher total diferulate content where two cycles of divergent selection gave a 16% higher diferulate concentration in stalk pith tissues than populations selected for lower diferulate content [77], suggesting that diferulate deposition in maize pith parenchyma cell walls is a highly heritable trait that is genetically regulated and can be modified through conventional breeding. Divergent selection also affected esterified diferulate and monomeric ferulate ether cross-link concentrations differently, supporting the hypothesis that the biosynthesis of these cell wall components is separately regulated [77].

Another example includes selection for ether-linked ferulates in smooth bromegrass (Bromus inermis), where eight clones were selected for divergence in ether ferulates from three out of four populations, which also showed independent variation for lignification, allowing the impact of ether ferulates and lignification on in vitro fiber digestibility (IVFD)
to be determined independently [78]. It was shown that divergent selection for ether ferulates or lignin levels had a significant effect on 96-h IVFD, reinforcing the importance of both the level of lignification and the amount of ferulic acid cross-linking lignin to polysaccharides via ether bonds in cell wall digestibility. Divergent selection for ether ferulate and lignin levels also allowed the association between these cell wall traits and pathogen resistance to be established.

6.1.2. Association and QTL Mapping

A number of studies involving quantitative trait loci or QTL mapping have contributed to the expansion of genomic regions, which participate in cell wall variation for hydroxycinnamic acid content and lignification, among other cell wall traits. Using progeny of a recombinant inbred maize line (RIL), QTLs affecting cell wall in vitro NDF (neutral detergent fiber) digestibility (IVNDFD), lignin content and composition, \( p \)-coumaric and ester- and ether-linked ferulic acid, as well as two diferulates, were identified [79]. The authors showed that ether-linked ferulic acid co-localized with IVNDFD QTLs, supporting the conclusion that ether ferulate levels, along with lignin levels, are involved in cell wall digestibility, and concluded that it is the capacity of ferulates to cross-link cell wall components that is the most relevant factor related to cell wall degradability where ferulates are concerned. The authors also suggested that breeding programs aiming at increasing cell digestibility could be improved by including \( p \)-coumaric acid and ether-linked ferulic acid together with IVNDFD traits. In a subsequent study, Barrière et al. (2012) [80] conducted QTL analysis in RIL progeny between two maize lines, and further expanded the number of QTLs for cell-wall-related traits. One QTL was demonstrated for ester-linked ferulic acid, one for ether-linked ferulic acid and seven for diferulates. Two of the diferulate QTLs were co-localized with IVNDFD QTLs, highlighting and supporting the previous study on IRL progenies [79] and the role of ferulate cross-linking in cell wall degradability.

In addition, the co-localization between ether-linked ferulic acid and IVNDFD QTLs reinforces the effect of cell wall ester-ether cross-linking hindering cell wall degradability. Interestingly, the ester-linked ferulic acid QTL was in a similar position to two PF02458 Arabidopsis putative feruloyl transferase genes (GRMZM2G050072 and GRMZM2G50270) [81], strengthening the likelihood that members of the CoA-dependent acyltransferase PF02458 family participate in AX feruloylation (to be discussed in more detail below).

With the aim of identifying candidate genes for agronomic traits (e.g., yield, DM, silking date) and feeding value related traits (e.g., IVCWD, lignin content), QTL analysis was conducted on maize RIL progeny developed from the cross between two lines, one of which was known for its high cell wall digestibility and silage intake [82]. Authors reported four QTLs for lignin content and two for cell wall digestibility; however, the lack of a systematic co-localization between lignin content and cell wall digestibility QTLs supports the known negative link between these two traits, and indicates, in addition, that variation in lignin content might be independent of cell wall digestibility. The RIL (R288 × F271) maize progeny together with co-localization studies have been used with the objectives of further investigating QTLs for lignin content and structure, hydroxycinnamic acid content and cell wall degradability, searching for underlying candidate genes and detecting possible hidden QTL positions [83]. Their findings showed that ether-linked ferulic acid and diferulate cross-links reduced cell wall degradability, while ester-linked ferulic acid had negligible effect, in agreement with previous results [78,82]. Substantial co-localizations were found between QTLs for cell wall degradability and both lignin structure and ether-linked ferulic acid and/or diferulate contents in the RIL progeny, leading the authors to suggest the possible involvement of a cluster of linked genes involved in cell wall biosynthesis and assembly.

A mapping population and genetic-marker-trait QTL association studies have been used to assess the genetic control of herbage quality traits (e.g., IVDMD, NDF, etc.) in perennial ryegrass (Lolium perenne) and to try to identify QTL-linked markers that could
be used for selection experiments [84]. As a result of this study, a total of 42 QTLs were detected for herbage quality traits, and a genomic region in *Lolium*, where herbage quality QTLs co-localize with lignin biosynthesis gene cluster, was also identified, and can be used as an important target for marker-assisted selection.

A genome-wide association studies (GWAS) technique, which allows a high-resolution mapping of QTLs associated with specific traits, has been used to identify, for instance, maize variations associated with specific cell wall components, such as cell-wall-bound ferulates. For example, using an association panel consisting of 368 diverse inbred lines grown across different environments, Wang at al. (2016) [85] performed a GWAS to identify single nucleotide polymorphisms (SNPs) associated with superior quality silage in mature maize stalk. The candidate genes found in this study, however, were mainly related to stress resistance and signal transduction, etc., except for one involved in cell wall biosynthesis. The authors concluded that the reason for the non-detection of favorable alleles involved in forage quality traits by the GWAS analysis lay in the fact that no silage maize was present in the association panel in the study.

López-Malvar et al. (2019) [86] were able to identify SNPs significantly associated with ferulic acid and total diferulates in maize pith using a subset of 282 inbred lines from a maize diversity panel and GWAS analysis. Although the effect of SNP association with hydroxycinnamic acids was found to be low, the authors identified candidate genes that could be involved in diferulate formation and in AX feruloylation, based on the number of SNPs for the respective traits.

6.1.3. Naturally Occurring and Induced Mutants

Ferulate Ester Mutants

Based on a *Mu* transposon-based screening program, Jung and Phillips (2010) [87] isolated a putative seedling leaf ferulate ester (*sfe*) mutant in maize (*Zea mays*) that showed an 8.8% reduction in leaf ferulate esters, a 2.2% reduction in mature sheaths and a 13.4% reduction in stems compared to the control W23 line. In fact, the impact of the *sfe* mutation was greater on ether-linked feruloylation of stems (−29.7%), leaf blades (−14.7%) and sheaths (−13.1%). Although the *sfe* mutants did not show a major phenotype change compared to the control W23 line, they were 3.8% taller, with a 7% increase in internode cross-sectional area and a 13% higher biomass after the tasseling stage. It was also shown that the *sfe* mutation led to an increase in cell wall AX in immature leaf blades and stems and a reduction in lignin deposition in immature leaves, mature sheaths and stems. The authors suggest that this mutation may have impacted the ferulate-AX complex rather than the ferulate ester synthesis alone. As predicted, there was a comparative increase in cell wall degradability for the *sfe* mutants at both the mature and immature stages of development. In a following study, Jung et al. (2011) [88] used the W23sfe maize mutants to evaluate the effect of reduced ferulate cross-linking on dry matter intake (DMI), milk production and in vitro digestibility in trials using lamb and cows compared to other corn lines including W22. The authors reported that cows fed diets containing W23sfe silages had higher DM intake and milk production and higher digestibility compared to the W22 silages. It was suggested that the lower levels of diferulates and ether-linked ferulate cross-links may have facilitated particle breakdown during silage chopping, resulting in smaller particles creating a higher cell wall surface exposure to microbial attack.

Brown Mid-Rib Mutants

Brown-midrib (*bmr*) mutant plants are characterized by a reddish-brown pigmentation of the leaf midrib and stems. They can originate from natural mutations or can be induced, and are most prominent in maize (*Zea mays*), sorghum (*Sorghum bicolor*) and pearl millet (*Pennisetum americanum*). The first *bmr* maize appeared in 1924 in a self-pollinated line of a dent maize [89]. The gene was named *bm1*, and three subsequent brown-midrib genes were described in maize (*bm2*, *bm3* and *bm4*). The brown-midrib mutations have all raised interest, because they have been shown to have a drastic effect on lignification
and hydroxycinnamic acid content, and as such, have provided an important and unique model for studying how these processes impact cell wall digestibility (see Cherney et al. 1991; Barrière and Argillier, 1993 for review) [90,91]. The cell wall composition of stems of bm mutants from sorghum (bm6 and bm18), pearl millet (bm) and maize (bm3) for ester- and ether-linked hydroxycinnamic acids and total lignin content were compared to their normal counterparts [92]. Their results showed a reduction in acid-insoluble lignin content, ether-linked ferulates and increase in the IVDMD of stems in all bm mutants, which indicated that the lower level of ether-linkages may also have a role to play in the increase in IVDMD. The variability in lignification and cell wall phenolics in the internodes of bm2 and bm3 in contrast with normal maize genotypes has also been evaluated [93]. Authors did not find any significant difference in the number of cell wall ester-bound ferulates between the three genotypes, but the effects on ether-linked ferulates were not reported. One factor that has been reported that is likely to complicate most of the studies on bm mutants is the different genetic backgrounds of the bm mutants and their wild-type counterparts. To overcome this problem, some researchers have used wild-type plants with the same genetic background as the bm mutants. In one of these studies, pyrolysis–mass spectrometry and pyrolysis–gas-chromatography–mass spectrometry were used to analyze the level of cell wall ferulates between a bm2 mutant and a wild-type with the same genetic background [94]. No major difference, however, was observed between the bm2 mutant and the wild type with respect to ferulic acid levels.

Four maize bm and a bm1–bm2 double mutant in near-isogenic backgrounds have also been studied for changes in cell wall structure and composition [95]. The authors reported differences in terms of bmrl mutational effects compared to previous studies in different genetic backgrounds, supporting the hypothesis that there are phenotypic differences among inbreds with bm mutations. The most striking changes observed in terms of cell wall composition were found in bm3 which had higher levels of ferulate monomers but similar level of AX cross-linking, indicated by similar amounts of ferulate dimers to wild type plants, which would support the view that the bm3 gene is not involved in ferulic acid biosynthesis. The double bm1-bm2 mutant was the only line to show a reduction in the total levels of diferulates compared to wild-type, indicating less AX cross-linking in the mutant. The double bm1-bm2 and the bm1 mutants showed equivalent levels of total (ester- and ether-linked ferulates), but lower levels of ether-linked ferulates than the wild-type, suggesting that a compensation mechanism is likely to have taken place. All the other bm mutants (bm2, bm3, bm4) showed an increase in the level of ether-linked ferulates compared to those of their wild-type counterparts [95].

Comparative analysis of stems for ester- and ether-linked ferulic acid content in mature control (F2, F292) and bmrl (bm1 to bm4) lines showed a significant reduction in ether-linked ferulate levels in bm1 and bm2 stems and a slight reduction in ester-linked ferulates in the bm1 lines only [96]. The fact that ferulic acid levels were not altered in the bm3 mutant led the authors to hypothesize that ferulic acid is not synthesized via caffeic acid O-methyltransferase (COMT), as it has been shown that bm3 mutants have low COMT activity [97].

6.2. Genetic Modification of Structural or Regulatory Gene Expression

6.2.1. Pathway Genes

Despite the importance of ferulates in different plant processes, as discussed above, the identification of putative genes coding for hydroxycinnamic acids incorporation into grass AX has been challenging. In addition, the precise mechanism by which ferulic acid is anchored to AX is still under debate. There has been only one report, regarding rice, which describes feruloyl transerase activity towards AX [98]. This work suggests that feruloyl: CoA: arabinoxylan-trisaccharide O-hydroxynnamoyl transferase catalyzes the transfer of ferulic acid to AX from feruloyl-CoA.

It is only over the past ten years that some candidate genes responsible for AX feruloylation have started to emerge. Feruloyl transerase (FT) candidate genes were first
proposed by Mitchell et al. (2007) [81]. Using bioinformatic analysis of expressed sequence tag (EST) libraries, the authors identified an orthologous group as candidate genes based on their higher expression levels in cereals compared to their orthologs in dicots. A family identified by this approach, originally comprising twelve genes, belongs to the betaine aldehyde dehydrogenase (BAHD) acyl-coenzyme A transferase superfamily (also known as the PF02458 family) (see D’Auria, 2006) [99]. Acylation mechanisms catalyzed by BAHD-acyltransferases are thought to be the cytosolic [99,100]. It has been proposed that feruloylation of AX takes place in the cytosol, having UDP-Araf as the acceptor for feruloyl-CoA substrate. UDP-Araf, which is generated in the cytosol [101], would be feruloylated by BAHD acyl-coenzyme A transferase and the Fer-Ara-UDP transferred into the Golgi, where the Fer-Ara group could be introduced into the generated AX via a feruloyl arabinose transferase [102–104].

The first study to assess the involvement of BAHD acyl-coenzyme A transferase identified by Mitchell et al. (2017) [81] in cell wall feruloylation was carried out in rice using the RNAi strategy [105]. The authors showed that downregulation of the transcription levels of four rice BAHD acyl-coenzyme A transferase genes (Os05g08640, Os01g09010, Os06g39470, Os06g39390) resulted in some reduction in the level of cell wall ester-linked ferulate monomers in leaves (up to 19%). Downregulation of FT gene(s) would be expected to reduce ferulic acid deposition on AX and consequently affect the level of cell wall ferulate monomers and dimers. Their results, however, lacked the quantification of ferulate dimers.

The expression of these BAHD acyl-coenzyme A transferase candidate genes and the level of ester-linked ferulic acids and p-coumaric acid in different tissues and at different developmental stages in Brachypodium distachyon has been described by Molinari et al. (2013) [106]. Although the evidence given by this study does not demonstrate the functionality of these genes, it certainly shows that the relative amounts of ferulic acid and p-coumaric acid are compatible with BAHD acyl-coenzyme A transferase gene expression levels.

A subsequent study [107] showed that overexpression of the OsAt10 (LOC_Od06g3990) gene in rice resulted in increased levels of matrix polysaccharide-bound p-coumaric acid, indicating the matrix polysaccharide transferase activity of putative OsAt10 in leaf blades, sheaths and mature straw. Further evidence for the involvement of BAHD acyl-coenzyme A transferase genes in AX feruloylation comes from Buanaﬁna et al. (2016) [108], who showed that altering the expression of the putative FT gene Bradi2g43520 (BdAT1) in the BAHD acyl-coenzyme A transferase family (homologue of the rice FT gene Os01g42880) resulted in changes in ferulic acid monomers and dimers content in Brachypodium distachyon cell walls. Downregulation of BdAT1 in several independent Brachypodium RNAi:BdAT1 lines resulted in up to a 35% reduction in ferulate levels in stems and leaves, and in contrast, its overexpression resulted in up to a 58% and 47% increase in ferulate levels in leaves and stems, respectively, when compared to control plants analyzed over 2–3 generations of selfing. Overexpression of the OsAt10 gene in switchgrass (Panicum virgatum) (PvAt10) was shown to significantly increase the level of p-coumaric acid in green leaves of transgenic lines (up to 28%), but to significantly decrease p-coumaric acid in senesced leaves (by ~15%) compared to the wild-type control. The levels of cell wall ferulic acid also decreased (21%–28%) in green tissues, but to a lesser extent in senescing leaves. The authors also reported a 40% and 30% increase in sacchariﬁcation of green and senescing tissues, respectively [109].

Expression of OsAt10 in sorghum (Sorghum bicolor) resulted in increased levels of xylan acylation with p-coumaric acid, but no change in the total amount of cell wall p-coumaric acid. No major changes were also observed for the overall content of cell wall ferulates, but the lignin content was reduced in the transgenic lines with an improvement in saccharification [110]. Furthermore, overexpression of a sugar cane ScAT10 (which shares 80% identity with OsAt10) in maize resulted in up to a 75% increase in the total level of p-coumaric acid in senescence culms [111].

Interestingly, most of the reports on overexpression of putative p-coumaroyl-CoA AX transferase AT10 genes also show a decrease in cell wall ferulates (except in sorghum) [110]...
and enhancement of cell wall saccharification in the transgenic lines compared to the wild-type control [107,109–111]; however, the mechanisms need to be further elucidated.

Silencing of SvBAHD01 in *Setaria viridis* [112] and of SacBAHD01 genes in sugarcane [113] by RNAi resulted in decreases in cell wall ferulates by 60% in the SvBAHD01 RNAi lines, and by 50% in SacBAHD01 RNAi line 1 and 30% SacBAHD01 RNAi lines 2.2 and 2.4, respectively. The *Setaria* and sugarcane RNAi lines also showed increases in biomass saccharification efficiency of 40%–60% and 24%, respectively. In a subsequent study on *Setaria v.s*, Mota et al. (2021) [114] characterized the BAHD05 acyl-coenzyme A transferase by its downregulation by RNAi technology. They showed that SvBAHD05-silenced transgenic lines had decreased levels of AX-p-coumaric acid in leaves, stems and roots, and they hypothesized that SvBAHD05 is a p-coumaroyl coenzyme A transferase involved in acylating AX residues with p-coumaric acid in *S. viridis*. These findings support the role of BAHD acyl-coenzyme A transferase genes in codifying acyltransferase enzymes likely to account for hydroxycinnamic acids incorporation into grass AX. The identification of such genes is crucial, not only as targets for grass improvement aimed at the biofuel, animal and biorefinery industries, but for elucidating the cell wall feruloylation process in grasses.

In addition to the significance of BAHD acyl-coenzyme A transferase in cell wall feruloylation modification in different grass species as discussed above, the impact of the α-1,3-arabinosyltransferase (XAT1) in AX feruloylation has also been studied [115]. Xylan arabinosyltransferases (XAT) are members of the CAZY family GT61 involved in the transfer of arabinose side chains to the xylan backbone [116]. It was shown that suppression of *Triticum aestivum* (TaXAT1) by RNAi was followed by a reduction in cell-wall-bound ferulic monomers and dimers in the water-extractable xylan from wheat [115].

### 6.2.2. Transcription Factors

A detailed transcription regulatory program for secondary cell wall formation has been quite well established in dicots. As grasses display a different cell wall composition and morphology from dicots, a distinct secondary wall formation program from the one established for dicots is expected. More recently, transcription factors (TFs) involved in secondary cell wall formation in grasses have been reported [117]. Some of the MYB family of TFs investigated so far in grasses show activation of lignin or secondary wall-related cellulose synthase genes (see Rao and Dixon, 2018) [117]. The role of the maize ZmMYB167 TF in the transcriptional regulation of secondary cell wall formation was studied by assessment of the effects of ZmMYB167 overexpression in the two distinct grass model systems maize and *Brachypodium* [118]. The effect of overexpression of ZmMYB167 in the level of cell wall ferulic acid was not consistent among the four transgenic *Brachypodium* lines studied. However, lignin levels increased up to 13% and cell wall p-coumaric acid by up to 24%, but saccharification was reduced by up to 20%. Furthermore, ZmMYB167 overexpression affected plant growth. In maize, overexpression of ZmMYB167 increased the levels of cell wall ferulic acid (up to 38%), lignin (up to 13%) and cell wall p-coumaric acid (up to 52%). In contrast with *Brachypodium*, it did not have any significant effect on plant growth, but reduced saccharification by up to 20%.

### 6.3. Expression of Transgenes That Alter Feruloylation

#### 6.3.1. Microbial Ferulic Acid Esterases (FAE)

Ferulic acid esterases (FAEs) are a subclass of carboxylic acid esterases that hydrolyze ester bonds between hydroxycinnamic acids and CW polysaccharides [9]. Targeted expression of FAEs in plants and the efficiency of the process in hydrolyzing cell wall ester-linked ferulates and diferulates were first demonstrated in *Lolium multiflorum* and *Festuca arundinacea* [66,67]. In both studies, *Aspergillus niger* FAE type A (FAEA) expression was targeted to the vacuole under a constitutive promoter, with the release of the enzyme upon cell death, resulting in increased cell wall degradability measured by both end point IVMD and initial rates of in vitro gas production. Buanafina et al. (2010) [119] also demonstrated that
constitutive targeted expression of FAEA in *Festuca* directly disrupted ester bonds linking ferulates to cell wall polysaccharides (apoplast targeting) or by reduced feruloylation prior to their incorporation into the cell wall (endoplasmic reticulum or Golgi targeting). Plants with reduced levels of cell wall ferulates showed increased cell wall degradability and increased rates of cellulase-mediated release of sugars.

*A. niger* FAE has also been expressed in wheat grains targeted to the apoplast (DF) or endoplasmic reticulum (DFK), with the aim of improving wheat grain properties for the baking industry, as well as for animal feeding [120]. FAE expression resulted in seeds with reduced fresh weights (20% and 50% in the DF and DFK lines, respectively) that were sh runeled compared to control non-transformed grains. There was also a significant reduction of 30% and 10% in the level of ferulate monomers (in the DF and DFK lines, respectively), with no changes in the levels of diferulates, but an increase in AX deposition in the water-unextractable AX (WE-AX). It was concluded that plants are likely to respond to the reduction in ferulate monomers by increasing AX deposition. The pleiotropic effect of constitutive FAE expression was also reported by Buanafina et al. (2017) [121], where the authors demonstrated that repeated vegetative propagation via tillering of *Festuca* plants constitutively expressing Golgi- and apoplast-targeted AnFAE resulted in a new phenotype where plant growth and morphology were altered. Buanafina et al. (2020) [64] also showed that maize plants constitutively expressing apoplast-targeted AnFAE were dwarfed.

In comparison with monocots, constitutive *A. nidulans* FAE apoplast-targeted expression in the dicot *Arabidopsis thaliana* resulted in a 60% reduction in the level of ferulate monomers compared to wild-type plants, without any visible impact on plant development. Although plants showed increased sugar release following incubation with a cellulase cocktail, it was not significantly higher compared to controls. According to the authors, the lack of pleiotropic effect was probably because dicot plant cell wall ferulate cross-linking is more than 10-fold lower than in monocots [122]. In contrast, expression of *A. niger* FAEB targeted to different cell compartments in alfalfa [123] resulted in reduced levels of ester bonds, increases in lignin content, and a reduced IVDMD, following 6 h and 72 h incubation with ruminal fluid. However, pre-treatment with alkaline peroxide followed by incubation with commercial cellulase increased glucose release in transgenic lines compared to control plants, and the authors suggested that transgenic lines had a higher digestibility compared to control plants following lignin removal.

6.3.2. Other Cell-Wall-Degrading Enzymes Expressed in Plants Affecting Cell Wall Feruloylation

Microbial xylanases constitute another group of enzymes that have been expressed in plants as an attempt to increase cell wall degradability, and which have, in some cases, impacted the level of cell wall hydroxycinnamic acids. The first report of either constitutive or induced xylanase expression in forage grasses [124] showed that constitutive expression of a *Trichoderma reesei* endo-xylanase (XYN2) in the apoplast or Golgi in *Festuca arundinacea* resulted in the regeneration of only a small number of plants and an increase in the levels of cell wall ferulate monomers and dimers. In contrast, senescence-inducible XYN2 expression in the apoplast was not only higher, but did not affect plant growth compared to constitutive expression, and resulted in increased levels of cell wall ferulate dimers. These changes in cell wall composition led to decreases in tissue digestibility and cellulase-mediated sugar release. It was hypothesized that transgenic plants expressing XYN2 could trigger plant defense response (mediated by ethylene and H$_2$O$_2$) and the production of H$_2$O$_2$ (detected *in situ*) could lead to increased ferulateimerization.

In another study, a *Bacillus subtilis* endo-xylanase was expressed in wheat endosperm with the aim of improving wheat grain properties for baking and animal feeding [120]. It was shown that the endo-xylanase activity was increased in transgenic grains compared to the control; however, grains were shruneled and had decreased biomass (25–33%). Cell wall analysis showed a significant increase in the arabinose to xylose ratio (10–15%) in the water-unextractable solids (WUS), indicating that endo-xylanase digested the unsubstituted areas
of AX. Although there was no difference in the levels of total and monomeric ferulates, there was a significant increase in the level of ferulate dimers, indicating higher AX cross-link in transgenic grains. Several aspects in this work drove the authors to conclude that these changes in ferulate dimerization were likely to be working as a compensation mechanism to prevent changes in the amount of AX in the WUS.

Three microbial enzymes, *A. nidulans* [β-xyllosidase/α-arabinosidase (AnXA), feruloyl esterase (AnFA) and an acetylxyylan esterase (AnAXE)] and *Xanthomonas oryzae* α-L-arabinofuranosidase (XoAF), were expressed in the apoplast of the model dicot *A. thaliana* [122]. The authors showed that only AnFA expression affected cell wall ferulate monomer levels (up to a 60% reduction).

To assess whether in-planta co-expression of FAEA and XYN2 could improve post-harvest cell wall deconstruction, *Festuca arundinacea* constitutively expressing vacuole- or apoplast-targeted *A. s niger* FAEA, were retransformed with an apoplast-targeted and senescence-induced *Trichoderma reesei* β,1-4 endoxylanase (XYN2). It was demonstrated that plants expressing FAEA and XYN2 in the apoplast, with the lowest levels of cell wall ferulate monomers, ferulate dimers and AX, released 31% more sugars following cellulase digestion and showed a five percentage-point increase in in vitro dry matter digestibility. However, constitutive apoplast FAEA expression negatively affected plant growth and resulted in 71% reduction in total biomass [125].

6.3.3. Genetic Manipulation of Lignin Genes

Cell wall hydroxycinnamic acid levels have also been impacted in some cases by genetic modification of the genes involved in lignin biosynthesis. For example, while disrupting the cinnamyl alcohol dehydrogenase (*BdCAD1*) gene in *Brachypodium* had no effect on ester-linked ferulates, it did result in increased levels of ferulate cyclobutene dimers, heterodimers and diferulate derivatives [126]. These results are likely to be the result of lower lignin levels in these mutants, as lignin, in addition to protecting ferulic acid esters from photodegradation, is also involved in creating alkaline-resistant structures via its ether linkage to ferulate dimers. Interestingly, it was reported that the level of ferulate dimers ether-linked to a G, H or S lignin unit were lower in *BdCAD1* mutants compared to controls, indicating a decrease in lignin levels.

In another study, the expression of a *Corynebacterium glutamicum* 3-dehydroshikimate dehydratase in *Arabidopsis* cell walls, with the aim of reducing lignin content and increasing saccharification, was shown to affect the lignin biosynthetic pathway and reduce the degree of lignin polymerization, and resulted in a 1.8–2.9-fold reduction in cell wall ferulates, along with improved saccharification [127]. However, it was not shown which ferulate forms (monomers or dimers) were impacted, and the effect on ether-linked ferulate levels was not determined, but it is likely that reduced cell wall lignin-polysaccharide cross-linking could be responsible, at least in part, for the increased saccharification in these transgenic lines compared to controls.

7. The Role of Cell Wall Feruloylation

The specific role of cell wall feruloylation and ferulate oxidative coupling in different plant processes are being elucidated. Because ferulates cross-link the polysaccharides they are attached to, this results in the modification of the physicochemical properties of the cell wall, with subsequent impacts on cell-to-cell adhesion, wall assembly, internode elongation, cell wall strength, plant resistance to pathogens and herbivores and cell wall degradability.

7.1. Feruloylation Contributes to Cell-to-Cell Adhesion

A fundamental process required for the formation and maintenance of normal plant structures is the adhesion of plant cells to each other, which involves the cross-linking between polymers located between the two primary walls. Ferulate dimers have been shown to play a significant role in cell-to-cell adhesion in plants, such as in Chinese water chestnut (CWC) [128] and beet root (*Beta vulgaris*) [129]. Parker and Waldron (1995) [128]
demonstrated that cell separation in CWC, which is not achieved by thermal treatment, could be induced by cold alkali treatment, which was shown to release ferulates and ferulate dimers before loss of tissue strength. Cold alkali treatment is known to hydrolyze esterified ferulates and diferulates to AX. These results provided further evidence for the involvement of diferulates in the CWC characteristic texture, and accounts for the thermal stability of cell–cell adhesion. The importance of beetroot cell wall ferulates in the thermal stability of cell adhesion was also investigated by Ng et al. (1998) [129]. They showed that vortex-induced cell wall separation would only take place after plant material was treated with cold alkali (0.05–0.1 M KOH), known to release ester-linked ferulates from the cell wall, which was also followed by loss of wall autofluorescence. It has also been observed that constitutive apoplast-targeted FAEA expression in maize resulted in spongy rather than rigid internodes, which did fall apart during the sectioning process compared to rigid and easier-to-section internodes from control non-transformed plants. Toluidine blue stained sections showed that the highly thickened sclerenchyma walls were replaced by thin-walled cells in transgenic lines compared to controls. These observations may reflect reduced cell-to-cell adhesion, due the significant reduced levels of diferulates in those internodes [64]. These results, taken together, support the notion that ferulate cross-linking xylan polymers in AX and the arabinose of RG-I contribute to cell-to-cell adhesion.

7.2. Feruloylation Contributes to Cell Wall Strength and Growth Cessation

Ferulates dimerization contribute to cell wall strength by forming diferulate-AX bonds cross-linking previously separated AX chains and generating a tighter arrangement of fibers within the wall [130,131].

A decline in wall extension and increase in wall stiffness have been correlated with increases in the levels of ferulate deposition and ferulate dimerization. In oat coleoptiles, the level of ferulate and ferulate dimer deposition increased as the growth rate of the coleoptiles started to decrease [132]. Similar findings have been reported by Tan et al. (1991) [133], Azuma et al. (2005) [134] and Wakabayashi et al. (2012) [135], where the levels of cell wall ferulates and diferulates correlated with cell wall mechanical stiffness and cessation of elongation growth rate of rice coleoptiles. The biosynthesis of ferulic acid occurs via the phenylpropanoid pathway, where phenylalanine ammonia-lyase (PAL) plays a limiting role [136]. It has been shown in oat and rice shoots that the levels of cell wall ferulates are correlated with PAL activity and that the level of diferulate formation changes with ferulic acid levels [132,133].

Another important aspect in addition to cell wall feruloylation is the coupling of ferulates, catalyzed by cell-wall-bound peroxidase (CW-PRX), which is likely to be regulating the formation of diferulates, as proposed by Fry (1986) [130]. As shown by Wakabayashi et al. (2012) [135], the levels of diferulate in rice shoot cell walls increased from day 4 to day 6 in parallel with increases in CW-PRX gene expression and enzyme activity, indicating that the expression of the CW-PRX gene in rice shoots might control the formation of ferulate dimers, strengthening the cell wall and causing a decrease in the growth rate of coleoptiles. Likewise, it has also been shown that the formation of ester-linked ferulate dimers in Festuca arundinaceae cell walls was correlated with a reduction in leaf elongation rate [65].

In previous work, Buanafina et al. (2010) [119] used an experimental system where the level of ferulates could be manipulated during cell wall formation by the targeted expression of an A. niger ferulic acid esterase (FAEA to the apoplast and Golgi system to assess the role of ferulate cross-linking on plant growth. Results showed that reducing the level of ferulates and diferulates in Festuca arundinaceae during leaf development altered the growth of newly emerged leaves, increasing the maximum relative segmental elongation rate in FAEA-expressing leaves (at 22%) compared to control leaves (at 19%), and that this occurred closer to the ligula (at 9 mm) than in controls (at 12 mm). This increase could be due to lower levels of ferulates being incorporated into the wall, allowing cells to expand faster, supporting the role of ferulates on cell expansion.
The role of cell wall feruloylation during maize internode elongation has also been investigated by Buanafina et al. (2020) [64], using transgenic maize plants expressing FAEA targeted to the apoplast under the control of either a constitutive or an inducible promoter. It was shown that reducing the levels of ferulates and diferulates early during plant development (constitutive promoter) by FAEA expression resulted in semi-dwarf (40%–60% height reduction) to extremely dwarf (over 90% height reduction) plants compared to controls. In contrast, when FAEA was under the inducible *Lolium multiflorum See1* senescing promoter, it was shown that the levels of ferulates and diferulates in maize internodes were not altered until after they had passed the stage of fast internode expansion, and as such, there was no major impact on internode expansion and plant growth. These results strongly support the role of normal cell wall feruloylation in preliminary stages of cell and internode elongation.

In *Brachypodium*, the reduction in the level of cell wall ferulates and diferulates by targeted expression of FAEA to the apoplast under a constitutive promoter also affected plant development, resulting in up to a 56% reduction in total height compared to control non-transformed plants (data not published). Similar findings were reported by De Souza et al. 2019 [113], where one transgenic switchgrass line overexpressing the *OsAt10* gene with reduced levels of ferulates displayed reduced growth and total biomass compared with the wild type. In contrast to these findings, Jung and Phillips (2010) [87] reported that the progeny of a putative seedling ester (*sfe*) maize mutant with an 8.8% lower level of ferulate esters at the seedling stage compared to control exhibited no negative effect on plant development or biomass yield; however, ferulate levels were only 8.8% lower for *sfe* at seedling stage and did not differ in immature sheath and stem in the *sfe* compared with control. No effect was observed on vegetative growth of *OsAt10* [107] or *SvBAHD01* [112] overexpressing lines with reduced levels of ferulate monomers and dimers.

Taking these results into consideration, one can argue that the degree of ferulic acid deposition and cross-linking are tightly regulated via PAL and CE-PRX expression to allow cell expansion and growth. In fact, it has been suggested by Fry (1980) [137] that gibberellic acid, which regulates plant growth by suppressing the secretion of wall-rigidifying peroxidase, and thus allows for cell elongation and thus expansion. In addition, the synthesis and integration of newly synthesized polymers and their cross-link constitute an essential part of the growth process, as the cross-link provides cell walls with the required strength to resist the pressure put upon them during the growth process. As AX cross-linking by ferulates is the type of cross-link that dominates in grasses, it is expected to be essential for growth.

7.3. Feruloylation Contributes to Resistance to Pathogens and Herbivores

The cell wall provides the first barrier that pathogens encounter and must break down before they can gain access into the cells. An important mechanism used by pathogens involves the secretion of cell wall hydrolytic enzymes. Plant cell wall components are also key factors in plant resistance to the tearing action of mandibles [138], where it has been shown that more sclerophyllous tissues tend to be tougher [139]. Ferulic acid is the major hydroxycinnamic acid in grasses, quite abundant in the sclerenchyma cells [140] and instrumental in the cross-linking of AX, so it is likely that feruloylation plays a role in plant resistance by increasing cell wall strength. Distinct groups have reported the association between diferulate content and resistance against herbivores [141,142] and pathogens [143–145]. The relationship between diferulate formation and resistance has been investigated in oat leaves when infected with *Puccinia coronate* [144]. The authors showed that the formation of diferulates coincided with an increase in ionically bound cell wall peroxidase in response to pathogen infection, and postulated that cell wall diferulate formation may contribute to oat resistance by providing a barrier to pathogen penetration. Garcia-Lara et al. (2004) [141] reported that weevil (*Sitophilus zeamais*) susceptibility was negatively correlated with total diferulate levels in maize pericarp, and suggested that ferulate cell wall cross-linking could contribute to maize weevil resistance by strengthening
the pericarp cell wall. Using smooth bromegrass clones divergently selected for ether ferulate and lignin levels, Delgado et al. (2002) [146] showed that resistance to Puccinia coronate, a biotrophic fungus, was negatively associated with lignin and ether ferulate levels. Based on these findings, authors suggested that the presence of ferulates cross-linking AX and lignin is likely to inhibit fungal penetration. P. coronate uses cell wall degradative enzymes as part its infection mechanism, and it has been suggested that the efficiency of such enzymes secreted by Puccinia might be affected by an increase in lignin and ether ferulate levels, as occurs in ruminants’ digestion [78]. If these associations are confirmed, it could present a problem for plant breeders aiming to combine both rust resistance and high forage digestibility in the same variety.

The role of cell wall ferulates in pest resistance has also been supported by studies using maize populations selected for higher levels of cell wall ferulates and diferulates [147]. In this study, the authors showed that corn borer larvae (Ostrinia lubilalis) fed on maize pith stem tissues from maize populations with higher cell wall diferulate levels had reduced dry weight (30%-40%) compared to larvae fed from populations with lower cell wall ferulates. Although these results reached a common conclusion about the role of feruloylation, they were all based on correlations between the two processes.

Based on previous studies where the level of ferulates in maize pith and leaf sheaths were reported to be associated with resistance to the Mediterranean corn borer, Santiago et al. (2008) [148] investigated whether resistance to corn borer could be enhanced by an increase in the level of phenolics through various cycles of recurrent selection in a maize population (EPS12). Although the level of cell wall phenolics did not significantly change between cycles, the higher concentration of diferulates was found to be associated with shorter tunnel length and a reduced number of larvae per stem, supporting the hypothetical role of diferulates in maize resistance to the Mediterranean corn borer.

The reduction in the level of cell wall ferulates as a result of fungal FAE expression in leaves has also been reported to increase plant vulnerability to insects and pathogens. Using the same experimental system, previously described, where the levels of cell wall ferulates and diferulates were specifically reduced by apoplast- and Golgi-targeted expression of A. niger FAEA in tall fescue [119], the role of ferulate cross-link in plant resistance to the herbivore fall armyworm (FAW) (Spodoptera frugiperda) was studied [149]. It was shown that both the growth rate and food utilization of FAW increased when fed with leaves from transgenic plants, with reduced levels of cell wall ferulates and diferulates. It was hypothesized that reduced levels of cell wall cross-link in transgenic leaves are responsible for lower wall strength and toughness, enabling higher nutrient assimilation at lower costs (i.e., less energy per bite) and higher FAW growth rates. Using a similar approach, Reem et al. (2016) [150] also showed that An FAE expression in Brachypodium resulted in reduced levels of ferulate monomers and dimers and increased susceptibility to Bipolaris sorokiniana, and in Arabidopsis it resulted in a decrease in the levels of ferulate monomers and wall-associated extensins and lower resistance to Botrytis cinera. The authors suggested that the reduction in cell wall ferulate cross-link reduces cell wall strength and leads to reduced resistance against pathogen attack. In a following study, Swaminathan et. al. (2021) [151] demonstrated that co-expression of A. nidulans FAE and acetyl esterase in Arabidopsis restored plant resistance to B. cinera.

7.4. Feruloylation Is Modified by Abiotic Stress

Adverse environmental conditions, such as extreme temperatures, drought and soil salinity (abiotic stressors), constitute a constant threat to plants, as they can exert several detrimental effects, such as membrane disorganization and loss of enzyme activity and osmotic stress, which can result in turgor loss, along with generation of excess levels of reactive oxygen species (ROS). As cell division followed by cell expansion drives the elongation of individual cells and the growth of plant organs, which depend on two major factors namely growth-driven turgor pressure and cell wall extensibility [152], such abiotic stressors are expected to have a direct impact on plant growth and development.
Plants have developed a multitude of strategies to cope with undesirable conditions, including modification of cell wall architecture and alteration of metabolism, such as by the induction of redox reactions, to reduce levels of reactive oxygen species (ROS) and osmotic adjustment [15,153]. For example, it has been shown that maize plants, when under salt or water stress, can restore shoot turgor pressure, while leaf elongation is only partially recovered [154,155], indicating that the loss in cell wall extensibility can be accounted for by the reduction in cell elongation. However, the physiological mechanism of early leaf growth inhibition by water stress has been shown to differ among species [156]. As discussed previously, the presence of cell wall ferulates results in a decrease in cell wall extensibility, and the accumulation of cell-wall-bound phenolics as a response to abiotic stressors has been observed [157,158].

Aiming to assess whether free and/or cell-wall-bound FA may support leaf adaptation to drought stress in triticale seedlings, Hura et al. (2009) [158] identified a winter genotype (Lamberto) that exhibited a higher content of cell-wall-bound ferulic acid under drought and rehydration conditions. The observed higher ferulic acid levels in the Lamberto variety, as a response to water deficit, positively correlated with significantly more efficient photosynthetic apparatus, and the authors hypothesized that this increase in ferulates as a response to water deficit might be a protective mechanism that acts as a filter, reducing the level of UV radiation reaching the mesophyll cells and decreasing leaf growth at the cost of osmotic adjustment. In a following study, Hura et al. (2012) [159] assessed whether the level of cell wall ferulates could have a positive effect on triticale water status and productivity under water stress. They showed that the higher levels of cell wall ferulates in plants subjected to soil drought treatment during the tillering and flowering phases were significantly correlated with higher activity of the photosynthetic apparatus of flag leaves, and negatively correlated with lower values of the amount of energy dissipated from PSII, indicating a less deleterious effect toward the photosynthetic apparatus and the protective effect of ferulates. They also showed that the higher levels of cell-wall-bound phenolics correlated with higher leaf hydration, (higher relative water content (RWC) and osmotic potential for flag leaves), which suggested that water loss from the symplast can be prevented by an increase in saturation of the cell wall with phenolics. They did not, however, establish which specific cell wall phenolic increased in response to drought treatment.

Using salt-sensitive and salt-resistant maize genotypes, Uddin et al. (2014) [160] also showed that diferulic acids in the cell wall may contribute to the suppression of shoot growth in the first phase of salt stress in maize. Salt stress treatment severely affected shoot elongation, causing a significant reduction in shoot fresh weight in all genotypes, but this reduction was significantly higher in the salt-sensitive compared to the salt-tolerant genotype. Salt stress treatment resulted in increased concentration of cell wall ferulic acid and of ferulate dimers in the upper shoot elongating zone of the salt-sensitive genotype, supporting the hypothesis that the increase in AX cross-linking by ferulate dimers is likely to be involved in reducing cell elongation and consequently the plant biomass in salt-sensitive genotypes.

A number of other roles of cell-wall-bound ferulates in plant adaptation to environmental stressors have been suggested, such as reducing the use of synthesized carbohydrates as building blocks in cell wall synthesis by preventing cell wall hydrolysis by endogenous enzymes involved in loosening cell walls during growth [161], and contributing to the increase in cell wall hydrophobicity and consequently inhibiting the flow of water from the symplast, reducing cuticular transpiration [162]. In fact, our own preliminary data showed that the reduction in the level of cell wall ferulates in maize by the expression of a ferulic acid esterase resulted in a decrease in RWC in leaves compared to control non-transformed plants. Another significant role of cell-wall-bound ferulates in plant adaptation involves the possible role of ferulates acting as a filter against UV-B radiation, limiting its penetration in cells, and as such, reducing, among other things, the generation of ROS.
7.5. Feruloylation Contributes to Cell Wall Degradability

Different cell wall properties have been associated with low enzymatic hydrolysis of polysaccharides, reducing both feed utilization by ruminants and their conversion to ethanol. As such, it is of interest to identify and target cell wall characteristics that limit energy availability of forages used as animal feed or for biofuel production, and to improve lignocellulose biomass deconstruction.

Because of its abundance and cross-linking, lignification has traditionally been considered the major factor affecting the resistance of cell walls to digestion or conversion to ethanol. It has been shown that plants with reduced lignification-specific enzymes [163,164] or with spontaneous and chemically induced mutations [90] had reduced lignification and increased saccharification/digestibility. However, reduced agronomic fitness, such as disease resistance, vigor, etc. has also been reported for plants in which lignin levels were reduced [165,166].

Another important feature that can significantly contribute to recalcitrance of the cell wall against digestion is the carbohydrate composition and architecture. It has been suggested that, for example, hemicellulose side chain modification, aiming to reduce hydrogen bonds with cellulose, may make cell walls more degradable [167]. The presence of diferulate residues cross-linking AX chains and covalently linked to lignin by ether bonds has also now been recognized as one of the most inhibitory factors in cell wall digestion by ruminants and lignocellulose saccharification, and this is supported by different studies.

Hartley (1972) [168] was the first to show that a significant correlation existed between the levels of \textit{p}-coumaric and ferulic acids in the cell wall and the digestibility of perennial ryegrass. Further studies using maize cell suspension cultures and a model synthetic system to manipulate the levels of ferulates, diferulates and lignification have also shown that reducing ferulates, diferulates and ferulate-lignin cross-linking improves hemicellulose degradability independently of lignin concentration [69,169,170]. There is also further supporting evidence for the negative effect of ferulic acid cross-linking AX to lignin on cell wall digestibility and for the view that lignin alone cannot solely account for the low cell wall digestibility of grasses [171,172].

Casler et al. (2008) [173] tried to determine whether intensive selection could alter the genetic correlation between lignin and etherified ferulates. They evaluated thirty clones each of smooth bromegrass (\textit{Bromus inermis} Leyss), orchardgrass (\textit{Dactylis glomerata} L.), and reed canopy grass (\textit{Phalaris arundinacea} L.) for different traits, including NDF, lignin, ester- and ether-linked ferulates and IVNDFD, in a replicated field study over a two-year period. They found that the phenotypic correlation between lignification and etherified ferulates was reduced in smooth bromegrass only, but that these two components had a negative effect on IVDMD and were maintained during the maturation stages of all three grasses. Based on the evidence that lignin polymer growth proceeds from monolignol attachment to ferulates ester-linked to AX, indicating that cell wall development and lignification are highly coordinated, the authors concluded that partitioning the effects of specific cell wall components on digestibility is quite difficult.

Using the experimental system in which FAEA expression was targeted to the vacuole under a constitutive promoter, in \textit{Lolium multiflorum} and \textit{Festuca arundinaeae}, with the release of the enzyme upon cell death, Buanafina et al. (2006, 2008) [66,67] showed that using this approach, FAEA was efficient in hydrolyzing ferulates and diferulates esterified to the cell wall, which resulted in increased grass digestibility, measured by both end point IVDMD and initial rates of in vitro gas production. They also showed that constitutive targeted expression of FAEA to the apoplast, endoplasmic reticulum (ER) or Golgi could also disrupt feruloylation of the expanding cell wall, enhancing digestibility and increasing rates of cellulose-mediated fermentable sugar release in tall fescue leaves [119]. In a later study using \textit{Festuca} suspension cultures, where FAEA was targeted to the vacuole under the control of a heat shock promoter, or to either the apoplast or the ER under the control of a constitutive actin promoter, the authors showed that the transformed cell lines had
reduced levels of cell wall ester-linked ferulates and increased rates of cell wall digestibility, measured as initial rates of in vitro gas production [174].

Based all the studies on growth, pathogen resistance, cell wall degradability and cell adhesion described above, it can be argued that cell wall feruloylation is an important process in plants, especially in grasses, and that understanding and manipulating cell wall feruloylation can contribute to improving the more efficient utilization of plant cell walls in the agricultural, food and bio-ethanol industries.

Author Contributions: Conceptualization, M.M.d.O.B. and P.M.; writing—original draft preparation, M.M.d.O.B.; writing—review and editing, M.M.d.O.B. and P.M.; All authors have read and agreed to the published version of the manuscript.

Funding: Work on cell feruloylation was initially supported by the Biotechnology and Biological Science Research Council UK (grant numbers D10116 and 98/B2/D/04647 to PM) and by Genencor Inc. USA, and later support was provided to MB by the U.S. Department of Agriculture (USDA), grant number 2009-35318-0513, and the USDA-DOE Plant Feedstock Genomics Research Program, grant number DE-FG02-08ER64701.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Acknowledgments: The authors thank all the students, research technicians, postdocs, and collaborators at both PSU and IGER, who contributed to the studies the authors have so far pursued with the aim of understanding cell wall feruloylation, the genes involved and its genetic modification.

Conflicts of Interest: The authors declare no conflict of interest.

References


79. Barrière, Y.; Thomas, J.; Denoue, D. QTL mapping for lignin content, lignin monomeric composition, p-hydroxycinnaminate content, and cell wall digestibility in the maize recombinant inbred line progeny F838 × F286. *Plant Sci.* 2008, 175, 585–595. [CrossRef]


81. Courtial, A.; Méchin, V.; Reymond, M.; Grima-Pettenati, J.; Barrière, Y. Colocalizations between several QTLs for Cell Wall degradability and composition in the F288 × F271 early maize RIL progeny raise the question of the nature of the possible underlying determinants and breeding capacity for biofuel capacity. *Bioenergy Res.* 2014, 7, 142–156. [CrossRef]


89. Barrière, Y.; Argillier, O. Brown-midrib genes of maize: A review. *Agronomie* 1993, 13, 865–876. [CrossRef]


130. Fry, S.C. Diferulic and ferulic acid in the cell wall of *Oryza sativa* coleoptiles—Their relationships to mechanical properties of the cell wall. *Physiol. Plant.* 1990, 78, 1–77. [CrossRef]


133. Tan, K.-S.; Hoson, T.; Masuda, Y.; Kamisaka, S. Correlation between cell wall extensibility and the content of diferulic and ferulic acids in cell walls of *Oryza sativa* coleoptiles grown under water and in air. *Physiol. Plant.* 1991, 83, 397–403. [CrossRef]


149. Buanafina, M.M.d.O.; Fescemeyer, H. Modification of esterified cell wall phenolics increases vulnerability of tall fescue to herbivory by the fall armyworm. *Planta* 2012, 216, 513–523. [CrossRef] [PubMed]


