

Lignin Metabolic Engineering in Grasses for Primary Lignin Valorization

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Abstract

Lignocellulose biomass is indispensable for establishing sustainable societies. Trees and large-sized grasses are the major sources of lignocellulose biomass. Moreover, large-sized grasses greatly surpass trees in terms of lignocellulose biomass productivity. With an overall aim to improve lignocellulose usability, it is vital to improve lignin content and simplify lignin structures in biomass plants via lignin metabolic engineering. In this mini review, recent studies of lignin metabolic engineering of grass biomass plants mainly from the authors' research group are summarized, which includes characterization of lignocellulose properties of large-sized grass biomass plants and the augmentation of lignin content and simplification of lignin structures in grasses.

Keywords: Grass; Lignin; Metabolic engineering; Structural simplification; Upregulation

INTRODUCTION

Recently, the concept of the bioeconomy, which is “the production, utilization and conservation of biological resources, including related knowledge, science, technology, and innovation, to provide information, products, processes and services across all economic sectors aiming towards a sustainable economy”¹⁾, has spread worldwide. It aims to switch from fossil resources to sustainable biomass resources for various economic activities, by incorporating not only the idea of biotechnology, but also of global sustainability and renewability²⁾. In addition, at the 21st Session of the Conference of the Parties to the United Nations (UN) Framework Convention on Climate Change (COP21) in December 2015, a multilateral agreement on climate change control (the Paris Agreement) was adopted. It requires all participating countries to put forth their best efforts to reduce CO₂ emissions, with the aim of achieving both global warming countermeasures and economic growth³⁾. Thus, the importance of renewable resources and energy has increased over the past decade. Among the renewable resources and energy, biomass resources are essential, because they are the only resources that can supply organic compounds, including industrial feedstock and liquid fuels.

Lignocellulose biomass, which is composed of lignin, cellulose, and hemicelluloses, accounts for the highest proportion of renewable terrestrial biomass accumulated on earth, and is therefore indispensable for establishing a sustainable society. The biomass mainly consists of the secondary cell wall of vascular plants, including trees and grasses. The worldwide annual consumption of tree lignocellulose biomass is estimated to be about 2 billion tons⁴⁾. Trees are indispensable for producing of wood-based materials and paper, which account for half of the total tree lignocellulose biomass consumption, while the other half is for burning. A significant part of the tree biomass used for fuel

depends on natural forest logging ⁴). Therefore, a reduction of natural deforestation is strongly required for sustainable development in harmony with environmental conservation. Additionally, although wood fuel use mainly occurs in developing countries, fuel wood consumption in Japan has increased by approximately 10 times during the last two decades ⁵), indicating the significance of fuel-use biomass even in developed countries.

In contrast, large-sized grasses largely surpass trees in terms of lignocellulose biomass productivity ⁴). The production of lignocellulose biomass derived from non-tree sources, mainly grass biomass, is estimated to be about 3.6 billion tons per year ⁶). The lignocellulose biomass production of grass biomass plants reaches about 7 to 93 ton ha⁻¹ yr⁻¹, which is much higher than the lignocellulose biomass production of trees (about 4 to 25 ton ha⁻¹ yr⁻¹) ⁴). Moreover, the lignin of grasses is generally easier to isolate than wood lignin ⁴). Taken together, to promote the future bioeconomic activities by efficiently using the limited area available for biomass production and the concomitant reduction of natural forest logging, the use of grass biomass will become increasingly important.

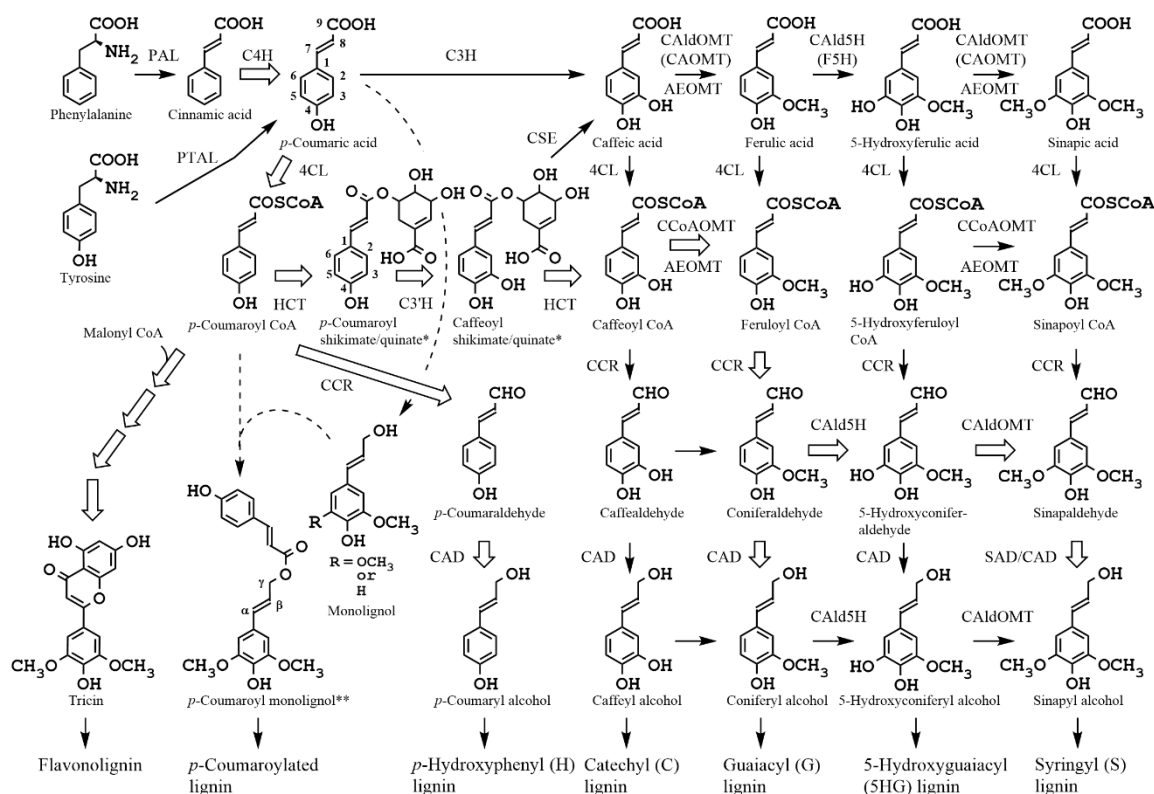


Fig. 1. The cinnamate/monolignol pathway in grasses.

Thick open arrow represents the major routes for lignin biosynthesis. The dotted arrow represents the novel and unknown pathway leading to *p*-coumaroylated monolignols. C4H, cinnamate 4-hydroxylase; C3H, *p*-coumarate 3-hydroxylase; CALD5H, 5-hydroxyconiferaldehyde *O*-methyltransferase; 4CL, 4-hydroxycinnamate CoA ligase; HCT, hydroxycinnamoyl CoA : shikimate/quinic acid hydroxycinnamoyl transferase; CCoAOMT, caffeoyl CoA *O*-methyltransferase; CCR, cinnamoyl CoA reductase; CALD5H, coniferaldehyde 5-hydroxylase; CAD, cinnamyl alcohol dehydrogenase; SAD, sinapyl alcohol dehydrogenase; F5H, ferulate 5-hydroxylase; CAOMT, caffeic acid *O*-methyltransferase; AEOMT, hydroxycinnamic acid/hydroxycinnamoyl CoA esters *O*-methyltransferase; C3'H, *p*-coumaroyl shikimate/quinic acid 3-hydroxylase; PAL, phenylalanine ammonia lyase; PTAL, phenylalanine/tyrosine ammonia lyase; CSE, caffeoyl shikimate esterase. Only the structures of *shikimate ester and ***p*-coumaroylated sinapyl alcohol are shown.



The chemical utilization of lignocellulose biomass can be classified into two categories: polysaccharide use and lignin use. Lignocellulose polysaccharides are encrusted by lignin, which has been considered an obstacle for the efficient use of polysaccharides during processes such as pulping, enzymatic saccharification, and forage digestion. Hence, numerous transgenic plants with reduced lignin content have been generated ^{4,7-9}). However, lignin is also a potential feedstock for aromatic products ^{7,9}), and is an important direct-combustion fuel or by-product fuel from polysaccharide utilization ⁴). For aromatic feedstock production, the complex structures of lignin make its utilization intractable. To address these difficulties, the simplification of lignin structures is an important breeding objective for future high-value-added, primary utilization of lignin ⁴). Higher lignin content is also beneficial for increasing the heating value of lignocellulose, because lignin has much larger heating values than polysaccharides ^{4,10}). The structural modification of lignin may also be an effective way to increase the heating values of lignocellulose biomass, because *p*-hydroxyphenyl (H) lignin has a slightly larger heating value, followed by guaiacyl (G) lignin and syringyl (S) lignin ⁴).

In this context, the present mini review summarizes recent reports from the authors' group, including the lignocellulose characterization of large-sized grasses, such as *Erianthus*, sugarcane (*Saccharum*) bagasse, and *Sorghum* with a view of using these materials to produce biomass fuel pellets and fermentable sugars ¹¹⁻¹³). It is noteworthy that the parenchyma-rich inner part of the *Erianthus arundinaceus* internode did not show a negative correlation between the enzymatic saccharification efficiency and lignin content ¹¹). In addition, to improve lignocellulose usability, metabolic engineering strategies were applied to the lignin biosynthesis of rice (*Oryza sativa* L. ssp. *japonica* cv. Nipponbare) as a model of large-sized grass biomass plants. Manipulation of expression of enzyme and transcription factor genes related to the lignin biosynthetic pathway, the cinnamate/monolignol pathway (Fig. 1) produced transgenic rice plants with decreased ¹⁴⁻¹⁶) and increased ¹⁷⁻¹⁹) lignin content, structurally modified lignins ²⁰⁻²²), and with improved biomass usability ²³).

Lignocellulose Properties of Grasses

Yamamura et al. characterized a large-sized grass, *E. arundinaceus*, in terms of lignins, *p*-hydroxycinnamic acids, and enzymatic saccharification efficiencies. The ratio of S to G lignins ranged from 0.43 to 0.79 and the lignin content ranged from 20% to 28%, with the highest values in the outer part of the internode. The amounts of ferulic acid formed after alkaline hydrolysis of cell walls were similar among all four organs (7.3–11.8 mg g⁻¹ dry weight of cell wall material), while there was more *p*-coumaric acid in the inner part of the internode (44.7 mg g⁻¹ dry weight of cell wall material) than in other organs (24.1–28.8 mg g⁻¹ dry weight of cell-wall material). The enzymatic saccharification efficiency of the leaf blade was 21.6%, while those of the other organs ranged from 10.0% to 15.2%. Of course, a huge number of papers have been published dealing with enzymatic saccharification of lignocellulose materials, and it is well known that higher lignin contents lead to lower in vitro digestibility or enzymatic saccharification efficiencies ⁴). Interestingly, however, there was an exceptional instance in which the parenchyma-rich inner part of the *E. arundinaceus* internode did not show a negative correlation between the enzymatic saccharification efficiency and lignin content ¹¹). In addition, the parenchyma-rich inner part of the *E. arundinaceus* internode showed distinct behavior in alkaline delignification: G lignin would be dominant in an alkaline-soluble fraction in the inner part of *E. arundinaceus* internode ¹²); by contrast, S lignin was likely dominant in an alkaline-soluble fraction in the outer part of the *E. arundinaceus* internode along with both parts of the sugarcane (*Saccharum* spp.) internode ¹²), as generally observed in alkaline delignification of grass lignocelluloses such as bamboo (*Bambusa rigida*) ²⁴) and *Sorghum bicolor* ²⁵) lignocelluloses. Overall, alkaline treatment removed lignins more efficiently from sugarcane tissues than from *Erianthus* tissues, resulting in a higher enzymatic digestibility of sugarcane tissues compared with *Erianthus* tissues ¹²). Taken together, these results showed that the characteristics of lignin and related compounds varied largely with species, tissues and organs of the large-sized grass plants ^{11,12}).

Furthermore, to improve the usability of lignocellulose materials by reducing lignin content, it is critically important to understand supramolecular structures of lignocellulose. However, largely because of the technical challenges in characterizing the highly complex and heterogeneous structure of lignocellulose, the supramolecular structure of lignocellulose and, consequently, its utilization



properties were not fully understood. In this regard, characterization of lignocellulose materials with altered chemical structures produced by metabolic engineering is a powerful strategy to understand lignocellulose supramolecular structures. However, only a few studies have attempted to analyze the supramolecular structure of lignocellulose in lignin-related mutants and transgenic plants²⁶⁻²⁸). Numerous reports were published dealing with downregulation/knockout of genes encoding enzymes on the cinnamate/monolignol pathway^{8,9}), though most of the works aimed at reduction of lignin content and improvement of lignocellulose polysaccharide usability. In line with this, Koshiba et al. manipulated the expression of genes encoding enzymes in the cinnamate/monolignol pathway which produce lignin monomers (Fig. 1)²⁹) by using *O. sativa* L. ssp. *japonica* cv. Nipponbare as a model for large-sized grass biomass plant¹⁴⁻¹⁶). First, the knockout effect of *CAD2* (*cinnamyl alcohol dehydrogenase2*) on rice lignin structures was examined. A *cad2* null mutant isolated from retrotransposon *Tos17* insertion lines of *O. sativa* L. ssp. *japonica* cv. Nipponbare exhibited brown-colored midribs in addition to hulls and internodes, clearly indicating both *brown midrib* (*bm*) and *gold hull and internode* (*gh*) phenotypes¹⁶). This is the first example of a *bm* phenotype in a C3 grass plant. The culm of the *cad2* null mutant showed 16.1% higher enzymatic saccharification efficiency than that of the control plants. The lignin content of the *cad2* null mutant was 14.6% lower than that of the control plants. Thioacidolysis of the *cad2* null mutant indicated the presence of cinnamaldehyde structures in the lignin. Taken together, these results showed that the deficiency of *OsCAD2* caused the *bm* phenotype in addition to *gh*. The brown coloration was probably due to the accumulation of cinnamaldehyde-related structures in the lignin¹⁶).

In addition, the knockdown effect of *OsCOMT1*, the gene encoding caffeic acid *O*-methyltransferase1 (= 5-hydroxyconiferaldehyde *O*-methyltransferase1, *CALdOMT1*) was also examined¹⁵). *COMT* genes are well known as another gene responsible for *bm* phenotype in some grasses such as maize and *Sorghum*^{30,31}). RNA interference (RNAi)-induced rice transformants with downregulated expression of *OsCOMT1* exhibited weakened cell wall staining with the Wiesner reagent in vascular bundle cells and sclerenchyma tissue compared with wild-type (WT) plants. This result suggested decreased lignin content, which was confirmed by wet chemical analysis. The lignin content decreased and the S lignin content drastically decreased in the transformants compared with the WT. However, unlike the *OsCAD2*-deficient mutant¹⁶), the *OsCOMT1* (*OsCALdOMT1*) knockdown did not cause clear *bm* phenotype. The transformants showed higher enzymatic saccharification efficiency up to 123.1% for the culm compared with that of WT, strongly suggesting that the transformant has an altered lignocellulose supramolecular structures¹⁵). These results were consistent with the fact that lignin generally inhibits the enzymatic saccharification process, although other factors also affect the enzymatic saccharification of polysaccharide fractions of grass lignocellulose¹¹).

Next, the *OsCAD2*-deficient mutant was subjected to characterization of lignocellulose supramolecular structures, which suggested that the *CAD2*-deficient lignocellulose had less well-defined cellulose alignment compared to that in the wild-type control lignocellulose³²). This result may account for the improved saccharification performance of lignocellulose produced by the *CAD2*-deficient mutant rice^{16,32}). In addition, single-knockout mutant lines that were deficient in *OsCALdOMT1* and double-knockout mutant lines that were deficient both in *OsCALdOMT1* and *OsCAD2* were generated from wild type and the *OsCAD2*-deficient mutant¹⁶), respectively, by *OsCALdOMT* mutagenesis using the Cluster Regularly Interspaced Short Palindromic Repeats (CRISPR)/CRISPR associated 9 (CRISPR/Cas9) system³³). Isolated homozygous mutant lines were subjected to in-depth characterization of the lignocellulose supramolecular structures. Though the disruption of *OsCALdOMT1* and *OsCAD2* evidently affected the supramolecular structure, the disruption of *OsCALdOMT1* more prominently affected the lignocellulose supramolecular structures than the disruption of *OsCAD2* did, resulting in higher cellulose mobility as primarily gauged by nuclear magnetic relaxation, at least for both mutants cultivated under employed conditions. Partly in line with this observation, *OsCALdOMT1*-deficient mutant lignocellulose showed significantly greater glucose release upon enzymatic saccharification compared to the wild-type control and the *OsCAD2*-deficient mutant lignocellulose³³). Although the saccharification efficiencies of the double-knockout mutant cell walls were improved compared with the performance of the wild-type control, the efficiencies appeared to be intermediate between those of the *OsCALdOMT1* and *OsCAD2* single-knockout mutant cell walls³³). Overall, these data supported the notion that genetic manipulation of lignin biosynthesis can



substantially affect the supramolecular organization of lignocellulose polymers in cell walls and thus eventually influences their deconstruction and utilization behaviors. Moreover, these mutants and transformants were also valuable for basic research to elucidate functions of lignins associated with plant physiology. Indeed, studies using the above mentioned transgenic rice plants demonstrated that lignin aromatic composition is an important factor affecting plant resistance to injurious parasitic plants^{34,35}.

Augmentation of Lignin Content

Breeding approaches to enrich lignins in biomass could improve the biorefinery process and solid biomass fuel use. This is because lignins represent a potent source of valuable aromatic chemicals and have larger heating values than polysaccharides^{4,10}). Recently, augmentation of lignin content in transgenic rice (*O. sativa* L. ssp. *japonica* cv. Nipponbare) using two strategies was reported; one was the heterologous expression and endogenous overexpression of transcriptional activator genes^{17,36,37} and the other was the suppression of endogenous transcriptional repressors^{18,19}.

Heterologous expression of three *Arabidopsis thaliana* (*Arabidopsis*) MYBs (*AtMYB55*, *AtMYB61*, and *AtMYB63*) resulted in rice culms with increased lignin content (about 1.5-fold higher than that in control plants)¹⁷). The lignin structures in *AtMYB61*-expressing rice plants were investigated by wet-chemistry and two-dimensional nuclear magnetic resonance spectroscopy (2D-NMR) approaches. The results suggested that heterologous expression of *AtMYB61* in rice increased the lignin content mainly by enriching S units as well as *p*-coumarate and tricetin moieties in the lignin polymers¹⁷). Studies with *S. bicolor* aiming to increase energy content in biomass plants were also reported; lignin content was augmented by overexpression of a *S. bicolor* transcriptional activator, SbMYB60, in *Sorghum*^{38,39}). In addition, lignin content of 30 Indonesian *S. bicolor* accessions was analyzed and high lignin-content accessions were identified¹³).

Targeted mutagenesis of the transcriptional repressor *OsMYB108* using CRISPR/Cas9-mediated genome editing generated lignin-enriched transgenic rice¹⁸). The *OsMYB108*-knockout rice mutants showed increased expression of lignin biosynthetic genes and enhanced lignin deposition in the culm cell walls. Chemical and 2D-NMR analyses revealed that the mutant cell walls were preferentially enriched in γ -*p*-coumaroylated and flavonolignin units (Fig. 1), both of which are typical and unique components of grass lignins. The results of an NMR analysis showed that the relative abundances of the major lignin linkage types were altered in the *OsMYB108* mutants¹⁸). Other putative transcriptional repressors, *OsWRKY36* and *OsWRKY102*, were also targeted using CRISPR/Cas9-mediated genome editing¹⁹). Both *OsWRKY36* and *OsWRKY102* mutations significantly increased lignin content by up to 28 % and 32 %, respectively. Additionally, *OsWRKY36/OsWRKY102*-double-mutant lines displayed lignin enrichment of cell walls (by up to 41 %). Chemical and NMR analyses showed that relative abundances of guaiacyl units and *p*-coumarate residues were slightly higher and lower, respectively, in the WRKY mutant lignins compared with those in the wild-type lignins, revealing that both *OsWRKY36* and *OsWRKY102* were associated with the repression of rice lignification. These results strongly suggested that the WRKYs and their close grass homologs are promising breeding targets for improving the utilization properties of grass biomass.

Simplification of Lignin Structures

The complexity of lignin structures has long been the challenging bottleneck in the utilization of lignin as an industrial aromatic feedstock⁴). To address this issue, lignin metabolic engineering aiming at simplification of the structures of lignin in rice (*O. sativa* L. ssp. *japonica* cv. Nipponbare) was reported by manipulating the expression of genes encoding enzymes involved in the aromatic ring modification shunts in the cinnamate/monolignol pathway (Fig. 1)²⁰⁻²²). Various studies have been published dealing with manipulation of expression of these genes^{8,9}). However, they aimed at characterization of gene functions and usability improvement of lignocellulose polysaccharides.

To increase S lignin content, rice transformants overexpressing *OsCald5H1* (CYP84A5), encoding coniferaldehyde 5-hydroxylase (Cald5H; = ferulate 5-hydroxylase, F5H), which is located in the S lignin biosynthetic shunt in the monolignol biosynthetic pathway (Fig. 1) were generated. A series



of wet-chemical and NMR structural analyses indicated that the S units were enriched by 2.3 fold in the rice transformants ²⁰⁾.

On the other hand, to increase G lignin content, an RNAi technique was used to downregulate the expression of *OsCald5H1* in rice. Compared with the control, the rice transformants produced altered lignins with a 1.2-fold enrichment of G lignin units ²⁰⁾. This result was further confirmed by a CRISPR/Cas9-mediated loss-of function experiment ²¹⁾. Homozygous *OsCald5H1*-knockout lines harboring the anticipated frame-shift mutations in *OsCald5H1* were analyzed using wet-chemical and 2D-NMR methods. The results confirmed that lignins in the mutant were predictably enriched in G units. Interestingly, however, lignins in all the mutant lines still contained considerable numbers of S units. A lignin γ -*p*-coumaroylation analysis by derivatization followed by reductive cleavage (DFRC) method revealed that the enrichment of G units in lignins of the mutants was limited to the non- γ -*p*-coumaroylated units, whereas the grass-specific γ -*p*-coumaroylated lignin units were almost unaffected. A gene expression analysis indicated that no homologous genes of *OsCald5H1* were overexpressed in the mutants. These data suggested that *Cald5H* is mainly involved in the production of non- γ -*p*-coumaroylated S lignin units, common to both eudicots and grasses, but not in the production of grass-specific γ -*p*-coumaroylated S units in rice ^{20,21)}.

The content of H lignin units was increased by downregulation and knock-out of the gene encoding rice *p*-coumaroyl ester 3-hydroxylase (*OsC3'H*) ²²⁾. The *OsC3'H*-knockdown rice lines generated via RNAi-mediated gene silencing, which showed about 0.5% of the residual gene expression levels, reached maturity and set seeds. In contrast, *OsC3'H*-knockout rice mutants generated via CRISPR/Cas9-mediated mutagenesis were severely dwarfed and sterile. Cell wall analysis of the mature *OsC3'H*-knockdown RNAi lines revealed that their lignins were largely enriched in H units (8 fold), while being substantially reduced in the normally dominant G and S units. Interestingly, however, the enrichment of H units was limited to non-acylated lignin units, with grass-specific γ -*p*-coumaroylated lignin units remaining apparently unchanged. The suppression of *OsC3'H* also resulted in a substantial reduction in the proportions of wall cross-linking ferulates. Collectively, the data demonstrated that *OsC3'H* expression is an important determinant not only of lignin content and composition, but also of the degree of cell wall cross-linking in rice. These results also showed that *OsC3'H*-suppressed rice displays enhanced lignocellulose saccharification efficiency ²²⁾.

These rice transgenic lines with distinct lignin monomer compositions (i.e., altered G/S/H aromatic unit ratios) were used to study the impact of lignin composition on the chemical reactivity, enzymatic saccharification efficiency, and calorific value of rice lignocellulose ²³⁾. The H-lignin-enriched rice transgenic line showed significantly enhanced biomass saccharification efficiency after alkali and acid pretreatments, and even without any pretreatment. The S-lignin-enriched rice transgenic line displayed enhanced saccharification efficiency after a hot water pretreatment. Although no significant differences in biomass heating values among the transgenic rice materials tested were observed, analyses of synthetic lignins comprising only G, S, or H units suggested that increased ratios of G or H units could increase the heating value of lignin-based solid biofuels ²³⁾. These strategies to increase lignin content and simplify lignin structures are also applicable to large-sized grass biomass plants, such as *Sorghum*, *Panicum* (switchgrass), *Miscanthus* and *Erianthus*. Recently, an increase in the energy content in biomass plants was achieved by the manipulation of *S. bicolor F5H (Cald5H)* expression ⁴⁰⁾ as well as the lignin content augmentation by *SbMYB60* overexpression in *S. bicolor* ^{38,39)} and isolation of high-lignin content *S. bicolor* accessions ¹³⁾.

Grass-specific Lignin Biosynthetic Pathways

As mentioned above, the *OsCald5H1*-overexpression ²⁰⁾, *OsCald5H1*-knockdown/knockout ^{18,19)}, and *OsC3'H*-knockdown ²²⁾ in rice did not produce completely single aromatic compositions, though these metabolic engineering efficiently modified the aromatic compositions to augment S-, G-, and H-units, respectively. This is in sharp contrast to the results of eudicot mutants or transformants. For example, *A. thaliana Cald5H*-deficient *fah1* mutants produced lignins most exclusively composed of G-units ^{41,42)}, while the *far1*-derived transformant in which *AtCald5H* was overexpressed made lignins from S-units ⁴²⁾. On the other hand, the *Arabidopsis C3'H*-deficient *ref8* mutant deposited essentially only H-units ⁴³⁾. Interestingly, the abundance of the G- and H-units of the *OsCald5H1*-deficient ^{20,21)}



is first converted to apigenin by flavone synthase II type CYP monooxygenase (OsFNSII, CYP93G1)^{61,62}. Apigenin is then oxygenated at the B ring by F3'H [CYP75B4 (and CYP75B3)] to give luteolin^{59,63}, followed by 3'-*O*-methylation of luteolin to chrysoeriol catalyzed by OsROMT9 (= OsCOMT1⁶⁴) and OsCaldOMT1¹⁵) or other OMTs⁵⁹, 5'-hydroxylation of chrysoeriol to selgin by CYP75B4⁵⁹, 5'-*O*-methylation of selgin to afford triclin by OsROMT9 (= OsCOMT1 and OsCaldOMT1)^{59,65}. Further, triclin is co-polymerized with monolignols to afford flavonolignin structures⁴⁹. Park et al. showed that recombinant OsCYP75B3 catalyzed both naringenin and apigenin hydroxylation, while recombinant OsCYP75B4 hydroxylated apigenin but not naringenin⁶⁶, which is in line with the above mentioned revised pathway (Fig. 2). Through characterizations of lignin and soluble flavonoid profiles in rice mutants deficient in CYP75B4 and CYP75B3, Lam et al. demonstrated that CYP75B4 alone provides sufficient 3',5'-hydroxylation for triclin-lignin (flavonolignin) deposition in rice vegetative tissues, whereas CYP75B3 is more responsible in a parallel pathway dedicated to the production of flavone *C*-glycosides⁶³. Lam et al. also demonstrated that cell wall lignins produced by *OsFNSII* (CYP93G1)- and CYP75B4-deficient rice mutants incorporate naringenin⁶² and apigenin⁶³ instead of triclin, which is in line with the proposed pathway for triclin biosynthesis (Fig. 2).

As early as 2006, *in vitro* methylation of luteolin and selgin by recombinant OsOMT1 (= OsROMT9) was reported^{64,67}. However, rice CCoAOMTs, OsROMT15⁶⁸, and OsROMT17⁶⁸ were found to methylate luteolin as well as caffeoyl CoA. These results indicate that several OMTs can catalyze *O*-methylation of luteolin in addition to OsROMT9. In contrast, in 2015 Lam et al. reported that OsROMT9-knockout rice mutant showed a 46% reduction in soluble triclin accumulation compared with wild type, while luteolin and selgin content increased; thereby, they concluded that OsROMT9 was a major OMT involved in triclin biosynthesis⁵⁹. This conclusion was confirmed by the biochemical characterization of recombinant OsCaldOMT1 (OsCOMT1, OsROMT9) and the chemical characterization of *OsCaldOMT*-RNAi knockdown transformants and T-DNA-inserted knockout lines, which contained largely reduced triclin-lignin (flavonolignin)⁶⁵. The latter was in line with the reduced levels of the triclin-lignin (flavonolignin) units in a maize mutant (*brown midrib3*, *bm3*) of *ZmOMT1*, a maize homolog of *OsCOMT1*, reported by Fornalé et al.⁶⁹ and in a *Sorghum* mutant (*brown midrib12*, *bmr12*) of *SbOMT*, a *Sorghum* homolog of *CsCOMT1*, reported by Eudes et al.⁷⁰.

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