Studies on the roles of 4-coumarate:coenzyme A ligase and 4-coumarate 3-hydroxylase in lignin biosynthesis in rice

（イネのリグニン生合成における 4-coumarate:coenzyme A ligase 及び 4-coumarate 3-hydroxylase の役割）

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Introduction

Lignin is a phenylpropanoid polymer produced in the secondary cell walls of vascular plants and comprises a large part of lignocellulosic biomass. Diverse metabolites derived from the cinnamate/monolignol pathway can serve as lignin monomers that combine to form complex lignin polymers via oxidative radical coupling in cell walls. In general, vascular plants including gymnosperms and angiosperms (both eudicots and monocots) utilize *p*-hydroxycinnamyl alcohols (monolignols) as common lignin monomers. On the other hand, monocotyledonous grasses utilize lineage-specific lignin monomers, such as monolignol *p*-hydroxycinnamate conjugates (mainly γ-*p*-coumaroylated monolignols) and tricin, a member of flavonoids, along with the canonical monolignols, for cell wall lignification (**Figure 1**). Because of the considerable interest in applying molecular breeding and bioengineering approaches to control lignin biosynthesis and increase the utility of the abundant grass biomass, the regulatory mechanisms of the cinnamate/monolignol pathway that coordinate the production of the diverse lignin monomers in grasses have been the focus of the related research.

In this study, the author investigated the functions of two key enzymes, i.e., 4-coumarate:coenzyme A ligase (4CL) and ascorbate peroxidase (APX)/4-coumarate 3-hydroxylase (C3H), in lignin biosynthesis using rice (*Oryza sativa*) as a model species for grasses. In particular, the roles of rice 4CL and APX/C3H isoforms in coordinating the production of lignin monomers derived from the cinnamate/monolignol pathway were investigated primarily by analyzing the cell wall structures of genome-edited rice mutants.

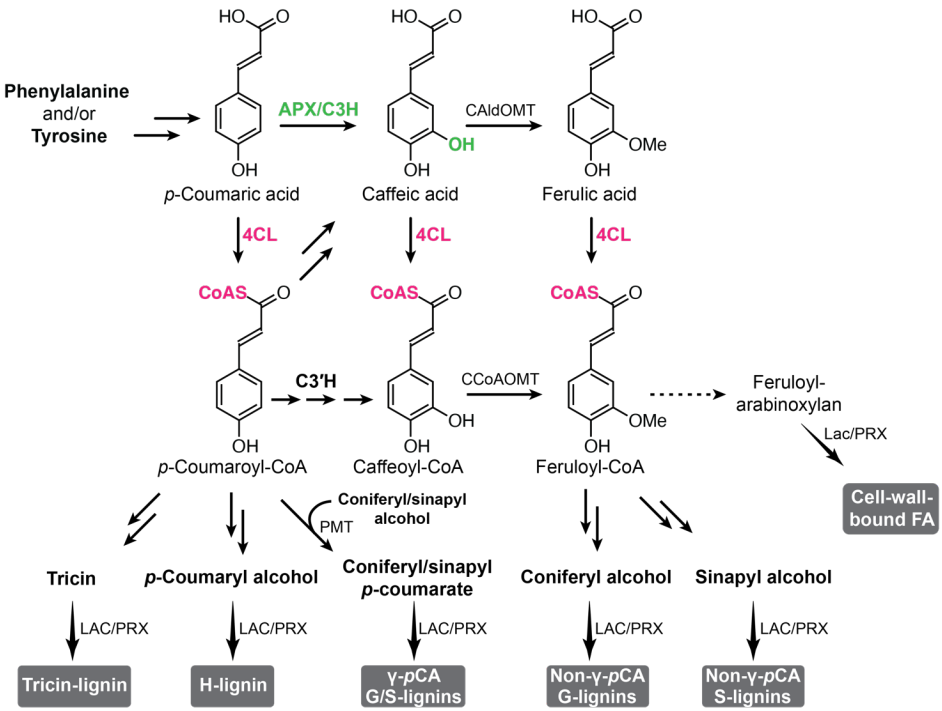

Figure 1. Proposed lignin biosynthetic pathways. The reaction steps catalyzed by 4CL and APX/C3H are highlighted

1. Divergent roles of two 4CL isoforms in the production of diverse grass lignin monomers

4CL is a key enzyme that contributes to channeling metabolic flux in the cinnamate/monolignol pathway by converting *p*-hydroxycinnamates into the corresponding coenzyme A (CoA) thioesters (**Figure 1**). Vascular plants often contain multiple 4CL genes. However, the contribution of each 4CL isoform to lignin biosynthesis remains unclear, especially in grasses. Here, the author characterized the functions of rice 4CL isoforms primarily by analyzing the cell wall chemical structures of genome-edited rice mutants deficient in the *4CL* genes.

Among five rice *4CL* genes, *Os4CL3* and *Os4CL4* were predominantly expressed in the lignifying rice culm tissues. Accordingly, rice mutants deficient in *Os4CL3* and *Os4CL4* were generated by CRISPR/Cas9-mediated targeted mutagenesis. In-depth cell wall structural analyses of the *4CL* mutants using a series of chemical methods and two-dimensional (2D) nuclear magnetic resonance (NMR) spectroscopy revealed that loss-of-functions of Os4CL3 and Os4CL4 differently altered the composition of lignin monomer units, demonstrating their diverse roles in lignin biosynthesis. As summarized in **Figure 2A**, the loss-of-function of Os4CL3 induced marked reductions in the major guaiacyl and syringyl lignin units derived from both the conserved non-γ-*p*-coumaroylated and the grass-specific γ-*p*-coumaroylated monolignols, with more prominent reductions in guaiacyl units than in syringyl units. In contrast, the loss-of-function mutation to Os4CL4 primarily decreased the abundance of the conserved non-γ-*p*-coumaroylated guaiacyl units, with little change in other monolignol-derived lignin polymer units. The loss-of-function of Os4CL4, but not of Os4CL3, reduced the grass-specific tricin-lignin units, indicating

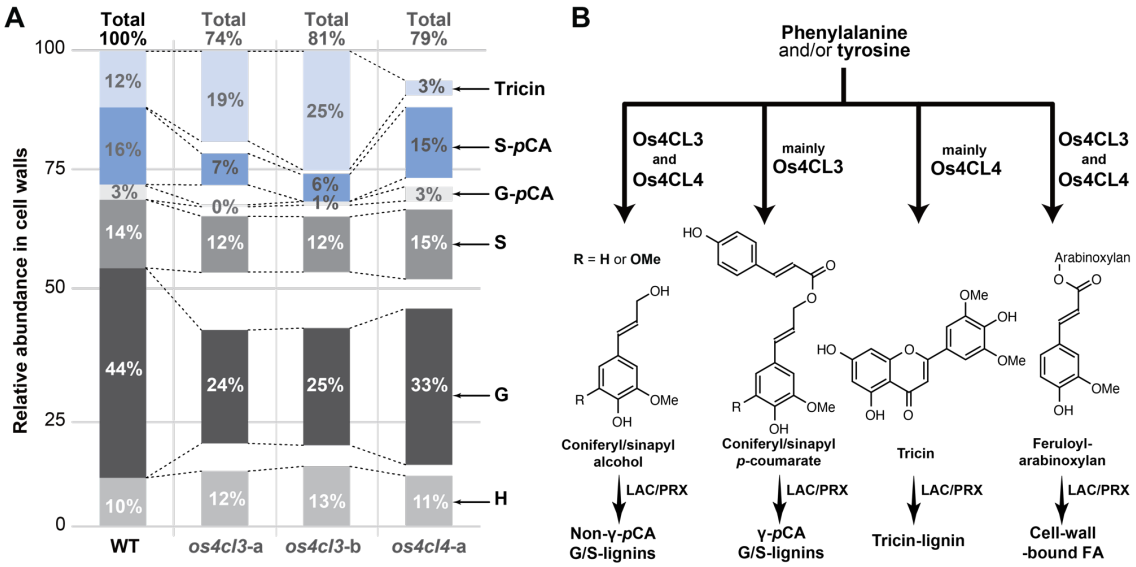

Figure 2. Summary of lignin compositional changes in *Os4CL3*- and *Os4CL4*-deficient rice culm cell walls detected in this study. Rough estimations of the relative abundances of major lignin polymer units in rice mutants culm cell walls (**A**) and proposed roles of Os4CL3 and Os4CL4 in lignin biosynthesis (**B**) are highlighted. Relative abundances of major lignin polymer units in rice culm cell walls were calculated based on derivatization followed by reductive cleavage (DFRC) and 2D NMR data of the rice cell walls.

that Os4CL4 plays a key role not only in monolignol biosynthesis but also in the biosynthesis of the flavonoid tricin used for lignification. Further, the loss-of-function of Os4CL3 and Os4CL4 notably reduced cell-wall-bound ferulates, indicating their roles in cell-wall feruloylation. Overall, these results demonstrate the overlapping but divergent roles of 4CL isoforms during the coordinated production of the diverse lignin monomers and cell-wall-associated ferulates in grasses (**Figure 2B**).

2. Role of APX/C3H in the biosynthesis of grass-specific lignin monomers

APX/C3H was recently identified as a bifunctional peroxidase that catalyzes the 3-hydroxylation of *p*-coumarate to caffeate (as C3H). Thereby, APX/C3H provides an alternative route to produce guaiacyl- and syringyl-type lignin monomers in the cinnamate/monolignol pathway apart from the conventional route in which a previously identified cytochrome P450 enzyme, *p*-coumaroyl ester 3-hydroxylase (C3′H), catalyzes the 3-hydroxylation of *p*-coumaroyl shikimate to caffeoyl shikimate (**Figure 3**). Further functional characterizations of grass APX/C3Hs are needed to establish their roles in producing the diverse lignin monomers in grasses and to understand the flux allocations between the parallel 3-hydroxylation pathways directed by APX/C3H and C3′H.

Protein sequence and gene expression analyses of rice APX enzymes identified two cytosolic APXs that may function as C3H, i.e., OsC3H1 and OsC3H2. Then, genome-edited rice mutants deficient in either or both *OsC3H1* and *OsC3H2* were generated by CRISPR/Cas9-mediated

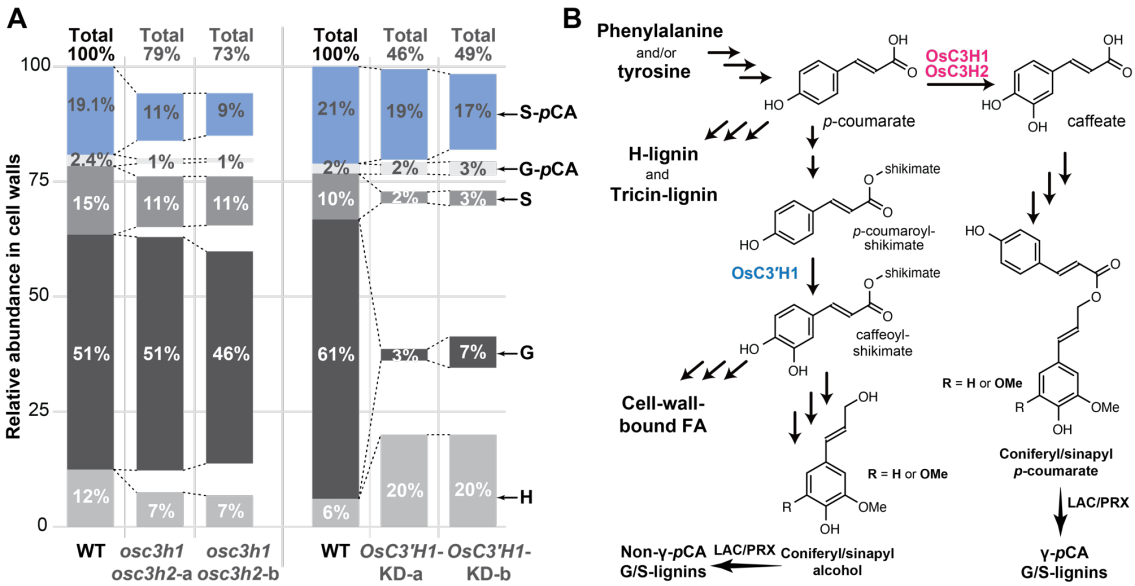

Figure 3. Summary of lignin compositional changes in *OsC3H1*- and *OsC3H2*-deficient rice cell walls compared with the coresponding compostional changes in OsC3′H-knockdown mutants (**A**) and proposed roles of OsC3H1 and OsC3H2 in lignin and cell-wall-bound ferulate biosynthesis (**B**). Relative abundances of major lignin monomer units in rice culm cell walls were calculated based on derivatization followed by reductive cleavege (DFRC). The compositions from OsC3′H-knockdown plants were retrieved from Takeda Y. et al., *Plant J,* 95, 796-811 (2018).

targeted mutagenesis. Lignin content analysis of the mutant cell walls detected significant lignin reductions in the cell walls of the double-knockout lines deficient in both *OsC3H1* and *OsC3H2*, but not in the single-knockout lines deficient in either of the two, indicating the redundant roles of OsC3H1 and OsC3H2 in lignin biosynthesis. In-depth cell wall structural analyses based on chemical and 2D NMR methods further revealed that such lignin reductions induced by *APX/C3H*-deficiency primarily occurred in the grass-specific γ-*p*-coumaroylated lignin units, with less prominent impacts on the contents of the conserved non-γ-*p*-coumaroylated lignin units (**Figure 3A**). These data suggest a preferential involvement of OsC3H1 and OsC3H2 in the biosynthesis of the grass-specific γ-*p*-coumroylated guaiacyl- and syringyl-type monolignols over the biosynthesis of the conserved non-γ-*p*-coumroylated guaiacyl- and syringyl-type monolignols. In contrast, it was previously demonstrated that downregulation of OsC3′H1 induced prominent reductions in the conserved non-γ-*p*-coumroylated guaiacyl- and syringyl-type lignin units, but not in the grass-specific γ-*p*-coumroylated guaiacyl- and syringyl-type lignin units, indicating the preferential involvement of C3′H in the biosynthesis of the non-γ-*p*-coumroylated guaiacyl- and syringyl-type monolignols over the biosynthesis of the γ-*p*-coumroylated guaiacyl- and syringyl-type monolignols (**Figure 3A**). Taken together, our data suggested that APX/C3H may direct the cinnamate/monolignol pathway flux preferentially toward the grass-specific γ-*p*-coumaroylated monolignols, complementing the conventional pathway leading to the conserved non-γ-*p*-coumaroylated monolignols via C3′H (**Figure 3B**).

Summary

1. The overlapping but divergent roles of Os4CL3 and Os4CL4 in lignin biosynthesis were demonstrated. Os4CL3 contributes more broadly to the biosynthesis of the major monolignol-type lignin monomers, i.e., both the conserved non-γ-*p*-coumaroylated and grass-specific γ-*p*-coumaroylated monolignols, whereas Os4CL4 contributes primarily to the biosynthesis of the non-γ-*p*-coumaroylated monolignols. In addition, Os4CL4, but not Os4CL3, plays a key role in the biosynthesis of tricin used for lignification.

2. The roles of cytosolic APX/C3H enzymes, OsC3H1 and OsC3H2, in lignin biosynthesis were demonstrated. OsC3H1 and OsC3H2 redundantly contribute to cell wall lignification, and they are preferentially involved in the biosynthesis of the grass-specific γ-*p*-coumaroylated guaiacyl- and syringyl-type monolignols, complementing the conventional cinnamate/monolignol pathway which leads to the conserved non-γ-*p*-coumaroylated guaiacyl- and syringyl-type monolignols via C3′H.