（ 続紙 1 ）

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論文題目	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）		

（論文内容の要旨）

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 the 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 esters (mainly γ-*p*-coumaroylated monolignols) and tricin, a member of flavonoids, along with the monolignols, for cell wall lignification. 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 related research areas. 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 the cinnamate/monolignol pathway in grasses using rice (*Oryza sativa*) as a model species. In particular, the roles of rice 4CL and APX/C3H isoforms involved in the production of lignin monomers were investigated primarily by analyzing the cell wall structures of genome-edited rice mutants.

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

4CL is a key enzyme that contributes to convert *p*-hydroxycinnamates into the corresponding coenzyme A (CoA) thioesters in the cinnamate/monolignol pathway. Vascular plants often contain multiple *4CL* genes. However, the contribution of each 4CL isoform to lignin biosynthesis remains unclear in grasses. Here, the author characterized the functions of two lignin-associated 4CL isoforms in rice, *i.e.*, Os4CL3 and Os4CL4, 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. The loss-of-function of Os4CL3 induced marked reductions in the content of the major guaiacyl and syringyl lignin units derived from both the 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 non-γ-*p*-coumaroylated guaiacyl units, with little change in other monolignol-derived lignin units. The loss-of-function of Os4CL4, but not of Os4CL3, reduced the grass-specific tricin-lignin units, indicating 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.

Taken together, 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.

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 and thereby suggested to be involved in an alternative route to produce guiacyl- and syringyl-type lignin monomers in the cinnamate/monolignol pathway apart from the conventional route in which a cytochrome P450 enzyme *p*-coumarate 3-hydroxylase (C3′H) catalyzes the 3-hydroxylation of *p*-coumaroyl shikimate to caffeoyl shikimate. Nevertheless, 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 (referred to as OsC3H1 and OsC3H2). Then, genome-edited rice mutants deficient in either or both *OsC3H1* and *OsC3H2* were generated by CRISPR/Cas9-mediated 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 *APX/C3H* genes, 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 content reductions induced by *APX/C3H*-deficiency in the *OsC3H1* and *OsC3H2* double-knockout mutant lines primarily occurred in the grass-specific γ-*p*-coumaroylated lignin units, with less prominent impacts on the contents of the non-γ-*p*-coumaroylated lignin units. These data suggest a preferential involvement of OsC3H1 and OsC3H2 in the biosynthesis of the grass-specific γ-*p*-coumroyrated monolignols over the biosynthesis of the non-γ-*p*-coumroyrated monolignols. In contrast, it was previously demonstrated that downregulation of OsC3′H1 induced prominent reductions in the content of the non-γ-*p*-coumroyrated lignin units, but not in the grass-specific γ-*p*-coumroyrated lignin units, indicating the preferential involvement of C3′H in the biosynthesis of the non-γ-*p*-coumroyrated monolignols over the biosynthesis of the grass-specific γ-*p*-coumroyrated monolignols. Taken together, the present study strongly suggested that APX/C3H is mainly involved the formation of the grass-specific γ-*p*-coumaroylated monolignols, complementing the conventional pathway leading to the non-γ-*p*-coumaroylated monolignols *via* C3′H.

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（続紙 ２ ）

（論文審査の結果の要旨）

イネ科植物のリグニンは、他の維管束植物のリグニンと比較して、より多様なリグニンモノマーから生成する複雑な化学構造を持つ。分子育種や代謝工学によるイネ科リグノセルロースの利用性向上に向けて、イネ科植物におけるリグニンモノマー生合成代謝経路の詳細な解明が求められている。本論文では、イネをモデル植物として用い、まず発現解析に基づきリグニンモノマー生合成経路上で働く *4CL* 及び*APX/C3H* の候補遺伝子を絞り込んだ。次いでこれらの候補遺伝子の機能を欠損したイネ変異株を作出し、そのリグノセルロース構造を詳細に解析することにより、多様なリグニンモノマー生合成に寄与する 4CL 及び APX/C3H アイソザイムを特定すると共に、それらの機能を明らかにした。評価すべき点は以下の通りである。

1. ゲノム編集を用いて、リグニンモノマー生合成に関与する *4CL* 及び*APX /C3H* 候補遺伝子の機能を欠損したイネ変異株を作出し、各種化学分析法と多次元NMR法を用いて改変されたリグニンの化学構造における差異を詳細に調べた。
2. イネの2つの4CLアイソザイム（Os4CL3 及び Os4CL4）がイネの多様なリグニンモノマー（非アシル化及びアシル化モノリグノール類ならびにトリシン）の生合成において、異なる機能を有することを明らかにした。
3. イネの2つのAPX/C3Hアイソザイム（OsC3H1 及び OsC3H2）が、イネ科植物に特有のリグニンモノマー（アシル化モノリグノール類）の生合成において、重複した役割を果たしていることを明らかにした。

以上のように、本論文は、イネ科リグノセルロースの利用性向上の基盤となる多様なリグニンモノマーの生合成制御機構の一端を明らかにしたものである。これらの成果は、植物代謝工学、植物二次代謝化学、木質科学及び農芸化学の基礎及び応用研究の発展に寄与するところが大きい。

よって、本論文は博士（農学）の学位論文として価値あるものと認める。

なお、令和５年２月１７日、論文並びにそれに関連した分野にわたり試問した結果、博士（農学）の学位を授与される学力が十分あるものと認めた。

また、本論文は、京都大学学位規程第１４条第２項に該当するものと判断し、公表に際しては、当該論文の全文に代えてその内容を要約したものとすることを認める。

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