Abstract of Thesis

Studies on Lignocellulose Supramolecular Structures and Deconstruction Properties in Lignin-altered Rice Mutants

（リグニンを改変したイネ変異体におけるリグノセルロースの超分子構造と分解特性に関する研究）

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Lignin is a complex phenylpropanoid polymer deposited in plant cell walls. Lignin has long been recognized as a key recalcitrant factor limiting the efficiency of lignocellulose deconstruction and downstream processing in polysaccharide-oriented biomass utilization processes, for example, those used in the production of pulp and paper and the generation of fermentable sugars for biofuels and biomaterials. To mitigate such lignin-associated biomass recalcitrance, numerous mutants and transgenic plants that produce lignocellulose with reduced lignin contents and/or lignins with altered chemical structures have been produced and characterized. However, largely because of the technical challenges in characterizing the highly complex and heterogeneous structure of lignocellulose, it is not fully understood how altered lignin chemistry affects the supramolecular structure of lignocellulose, and consequently, its utilization properties. This study therefore aimed to further dissect the impacts of genetic modifications of lignin on the supramolecular structure and deconstruction properties of lignocellulose. Particular focus was directed to the characterization of rice mutants deficient in *CINNAMYL ALCOHOL DEHYDROGENASE* (*CAD*) and *5-HYDROXYCONIFERALDEHYDE* O-*METHYL-TRANSFERASE* (*CAldOMT*), both of which encode key enzymes in lignin biosynthesis and represent major bioengineering targets for lignin modification (Figure 1).

In the first part of this study, the author conducted comprehensive chemical and supramolecular structural analyses of lignocellulose produced by a *CAD*-deficient mutant rice. The solution-state two-dimensional NMR approach and complementary wet-chemical methods elucidated the structural details of the altered lignins enriched with unusual hydroxycinnamaldehyde-derived subunits produced upon *CAD* deficiency (Figure 1). In parallel, lignocellulose supramolecular structure was investigated by solid-state ^{13}C magic-angle-spinning (MAS) NMR, nuclear magnetic relaxation, X-ray diffraction, and Simon's staining approaches. The obtained data indicated that polysaccharide assembly and mobility were notably disrupted in the *CAD*-deficient mutant lignocellulose, which may contribute to the improved biomass saccharification performance of the mutant compared to that of the wild-type rice control.

In the second part of this study, the impacts of lignin-modifications induced by deficiencies of *CAldOMT* and *CAD* on lignocellulose structures and properties were comparatively investigated. To this end, a series of rice mutants deficient in either or both rice *CAldOMT* and *CAD* genes were generated by CRISPR/Cas9-mediated targeted mutagenesis, and their lignocellulose chemical and supramolecular structures as well as enzymatic saccharification performance were comparatively evaluated. In line with the proposed functions of CAldOMT and CAD in the lignin biosynthetic pathway (Figure 1), the *CAldOMT*-deficient rice produced lignin largely depleted in syringyl and tricin subunits, whereas the *CAD*-deficient rice produced lignin incorporated with unusual hydroxycinnamaldehyde-derived subunits as demonstrated earlier. It was found that *CAldOMT* deficiency more prominently affects the lignocellulose supramolecular structure by resulting apparently less integrated polysaccharide assembly than does *CAD* deficiency. In line with this observation, the *CAldOMT*-deficient mutants displayed significantly higher enzymatic saccharification efficiency compared with that of the *CAD*-deficient mutants, although no synergistic improvement of the saccharification efficiency with disruptions of both *CAldOMT* and *CAD* genes was observed.

In the last, third part of this study, the author further characterized lignocellulose deconstruction properties of the *CAD* and *CAldOMT* mutant rice upon biomass processing reactions, i.e., dilute alkali, dilute acid and liquid hot water treatments. In-depth NMR and chemical structural analyses on rice lignocellulose before and after the chemical treatments revealed the different reactivities of various lignin subunits as well as polysaccharide components in *CAD*- and *CAldOMT*-deficient mutant lignocellulose. Notably, the saccharification efficiency of the mutant lignocellulose was largely improved over the wild-type control by applying the chemical treatments before subjecting to enzymatic hydrolysis of polysaccharides. The dilute alkaline treatment was effective to promote the saccharification of both *CAD*- and *CAldOMT*-deficient mutant lignocellulose, whereas the dilute acid and liquid hot water treatments were effective for *CAldOMT*-deficient mutant lignocellulose but not for *CAD*-deficient mutant lignocellulose. Overall, the use of the biomass processing reactions in combination with *CAD*- and *CAldOMT*-induced lignin modifications is strategic to further boost lignocellulose deconstructions for biorefinery purposes.

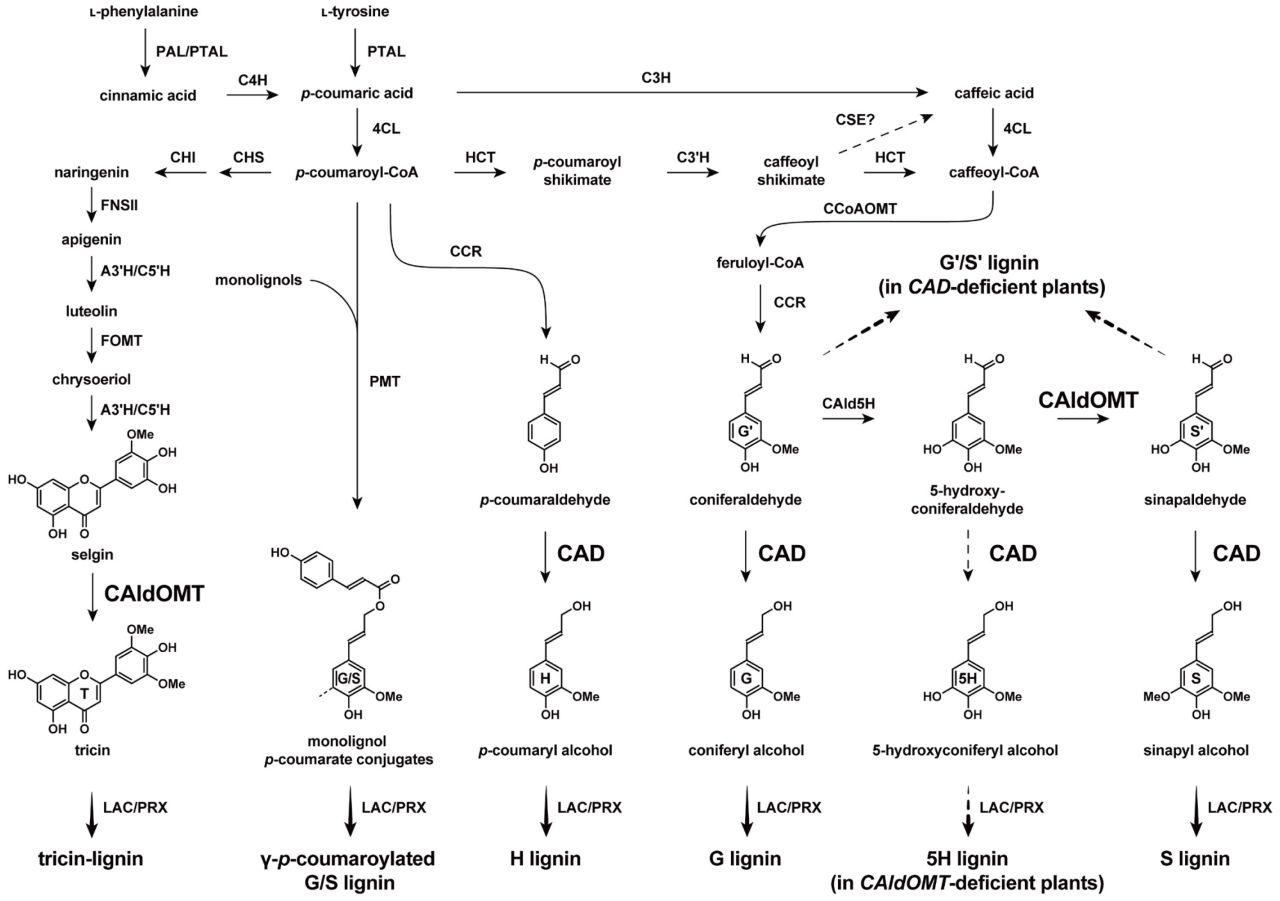

Figure 1. Proposed lignin biosynthetic pathways in grasses. Cinnamyl alcohol dehydrogenase (CAD) catalyzes the conversions of hydroxycinnamaldehydes to the corresponding hydroxycinnamyl alcohol (monolignol) precursors. 5-Hydroxyconiferaldehyde *O*-methyltransferase (CAldOMT) is a bifunctional enzyme that catalyzes the *O*-methylations of 5-hydroxyconiferaldehyde and selgin in the monolignol and tricin biosynthetic pathways, respectively. PAL, phenylalanine ammonia-lyase; PTAL, bifunctional phenylalanine tyrosine ammonia-lyase; C4H, cinnamate 4-hydroxylase; C3H, coumarate 3-hydroxylase; 4CL, 4-coumaroyl-CoA ligase; HCT, *p*-hydroxcinnamoyl-CoA: shikimate *p*-hydroxycinnamoyl transferase; C3′H, *p*-coumaroyl ester 3-hydroxylase; CSE, caffeoyl shikimate esterase; CCR, cinnamoyl-CoA reductase; CCoAOMT, caffeoyl-CoA *O*-methyltransferase; CAld5H, coniferaldehyde 5-hydroxylase; PMT, *p*-coumaroyl-CoA: monolignol transferase; CHS, chalcone synthase; CHI, chalcone isomerase; FNSII, flavone synthase II, A3′H/C5′H, apigenin 3′-hydroxylase/chrysoeriol 5′-hydroxylase; FOMT, flavonoid *O*-methyltransferase; LAC, laccase; PRX, peroxidase.