Characterization of *Oryza sativa* caffeate *O*-methyltransferase

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The low efficiency in the enzymatic saccharification is attributed to the fact that lignin tightly covers cellulose and hemicelluloses which are the substrates for hydrolases. Therefore, the regulation of lignin biosynthesis in biomass plants would improve the yield of the enzymatic saccharification. In this context, the elucidation of the biosynthetic mechanism of lignin biosynthesis in biomass plants is important.

Lignin is biosynthesized via the cinnamate/monolignol pathway. The pathway in dicotyledons has been extensively studied thus far. In contrast, the pathway in monocotyledons has not been well understood. Monocotyledons include important biomass plants such as switchgrass, erianthus, miscanthus, and sorghum classified as Gramineae. In this study, the author characterized a caffeate *O*-methyltransferase, a key enzyme involved in syringyl lignin biosynthesis in the cinnamate/monolignol pathway using rice, a model plant of Gramineae, and examined the increase of the saccharification by knock-down of the gene expression.

First, the author conducted the biochemical characterization of a rice *O*-methyltransferase (COMT3). Recombinant COMT3 was expressed in *E. coli* and purified as a His-tagged protein. The protein was reacted with various possible substrates, such as caffeic acid, 5-hydroxyferulic acid, caffealdehyde, 5-hydroxyconiferaldehyde, caffeyl alcohol, and 5-hydroxyconiferyl alcohol. After trimethylsilyl derivertization, the products were analyzed on GC-MS. V_{max}, K_m and k_{cat} values for each substrate (10-150 μM) were determined. As a result, COMT3 was found to catalyze *O*-methylation of all substrates. Among these substrates, 5-hydroxyferulic acid and 5-hydroxyconiferaldehyde showed smaller K_m (22 and 26 μM, respectively) and larger k_{cat} K_m^{-1} (478 and 294 μM^{-1} min^{-1}, respectively) than those of the other substrates, suggesting that the two substrates are the best two for COMT3 in this study. Furthermore, COMT3-mediated *O*-methylation of 5-hydroxyferulic acid was strongly inhibited by 5-hydroxyconiferaldehyde (competitive inhibition, K_i 2 μM), while that of 5-hydroxyconiferaldehyde was not inhibited by 5-hydroxyferulic acid. A substrate inhibition was observed when the concentration of 5-hydroxyconiferaldehyde was higher (25-150 μM). Interestingly, when 5-hydroxyferulic acid was added to the reaction mixture, the substrate inhibition was mitigated. Taken together, the results indicate that 5-hydroxyconiferaldehyde was preferentially methylated by COMT3 compared to 5-hydroxyferulic acid, strongly suggesting the pathway for syringyl lignin biosynthesis from coniferaldehyde to sinapyl alcohol *via* 5-hydroxyconiferaldehyde and sinapaldehyde in rice.

Second, the author conducted the characterization of COMT3-downregulated rice by RNA interference (RNAi) to examine the function of COMT3 *in vivo*. The T1 population of *COMT3* RNAi lines of rice plants was cultivated in a 3-L plastic container containing a nutrient solution in a growth chamber. The plants were submitted to quantitative reverse-transcriptase PCR (qRT-PCR) analysis of the gene expression, lignin staining, thioglycolic acid lignin analysis, and enzymatic saccharification. qRT-PCR analysis revealed that the expression of *COMT3* RNAi lines were strongly suppressed compared with wild type in all organs tested (leaf, sheath, and stem), indicating that the RNAi of *COMT3* in rice was successful. In Wisner staining, cortical fibers and vascular bundles were stained dark red in wild type, but were stained light red in the RNAi lines, suggesting that a decrease in lignin quantity in the RNAi lines. Thioglycolic acid lignin analysis showed that in wild type, lignin contents ranged from 14.9 to 22.6 %, whereas those in the RNAi lines, ranged 7.2 to 16 %, and these of all parts were decreased compared with wild type. The saccharification efficiencies of RNAi lines increased 2 to 12.2 % compared to wild type.

Taken together, this study strongly suggests the COMT3 preferentially catalyzes the formation of sinapaldehyde from 5-hydroxyconiferaldehyde and is involved in lignin biosynthesis, and the COMT3 down-regulation is effective for the increase of the efficiency of saccharification of rice straw.