Chinese Science Bulletin 2004 Vol. 49 No. 15 1602—1606

Characterization of three rice *CCoAOMT* genes

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Abstract **Caffeoyl-Coenzyme A 3-O-Methyltransferase (CCoAOMT) is a key enzyme in lignin biosynthesis pathway. Three rice *CCoAOMT* genes were identified and designated as *OsCOA1, OsCOA20* and *OsCOA26. OsCOA1* contains four exons and three introns, while the other two have three exons and two introns. The deduced amino acid sequences of these rice genes share a high identity (75.43%) with other plant CCoAOMT proteins and contain the CCoAOMT specific motifs. Phylogenetic analysis indicates that OsCOA1 has the closest evolutionary relationship to maize CCoAOMT. In contrast, OsCOA20 and OsCOA26 belong to another clade. Northern blot analyses and *in situ* hybridization studies indicate that the three rice *CCoAOMT* genes are highly expressed in developing sclerenchyma cells and vessel bundles of young leaves, suggesting that they are probably involved in constitutive lignification.**

Keywords: rice Caffeoyl-Coenzyme A 3-O-Methyltransferase, lignin.

DOI: 10.1360/04wc0225

Caffeoyl-Coenzyme A 3-O-Methyltransferase, a *S*-adenosyl-L-methionine (SAM)-dependent *O*-methyltransferase (OMT), transfers the methyl groups of SAM to positions 3 on the aromatic ring of monolignols and catalyzes caffeoyl-Coenzyme A into feruluyl-CoA[1]. It is a key enzyme for lignin biosynthesis, which is involved in the plant defense reaction by synthesizing wall-bound forms of ferulic acid[2,3]. Plant OMTs are divided into two families according to distinct substrate preference and amino acid sequence homology, namely PI-OMT Ⅰ and PI-OMT Ⅱ. Caffeoyl-Coenzyme A 3-O-Methyltransferas (CCoAOMT) belongs to the former family. Its molecular weight is smaller and the cofactor Mg^{2+} is required for enzyme activity. The OMT amino acid sequence contains eight conserved motifs. Motifs A, B and C are common in plant OMTs, while motifs D, E, F and G, H are CCoAOMT signature sequences[4].

CCoAOMT is a gene family in many plants, so are other enzymes in lignin biosynthesis[5]. The specific roles of each isoenzyme in plants are difficult to be determined because many potential substrates and multitude of isozymes exist in plants. Now many studies have directed towards the identification of CCoAOMT specifically involved in the process of lignification because there is great economic value in genetically modifying lignin biosynthesis by downregulating its expression to cultivate materials suitable for agro-industry utilization[6−9]. However, a great deal of researches on the lignin methylation pathway is mainly focused on dicots, while little is known of monocots. Rice is an important crop in the world. Characterization of rice genes involved in lignification will open up the possibility of improving the resistance of rice against lodging and forage digestibility by genetically modifying lignin biosynthesis. Here we report the gene structures and expression patterns of three rice *CCoAOMT* genes. Our studies indicate that the isolated *CCoAOMT* genes is likely involved in lignification in rice, which laid a foundation of further investigation into the lignin methylation pathways occurring in monocots and breeding lodging resistant varieties/lines of rice.

1 Method and materials

(ⅰ) Plant materials. Rice cultivar Zhonghua10 (*Oryza sativa ssp. japonica*) seeds were placed on water-soaked 3MM filter in a petri dish and kept in the dark at 28℃ for 2 d. Germinated seeds were transferred to the growth chamber (16 h of light/8 h of darkness) at 28℃ for 12 d. The tissues of 14-day-old seedling were selected for Northern blot analyses and *in situ* hybridization studies.

(ⅱ) Identification of the rice *CCoAOMT* genes. Chinese White poplar *CCoAOMT* (accession number AF240466) was searched against the predicted homologous sequences in rice GenBank provided by Shen Qingxi using the blast program. Three *CCoAOMT* sequences were identified. Their gene structures and corresponding predicted amino acid sequences were analyzed by Genscan program. To study the expression profile of these *CCoAOMT* genes, the primers were designed according to the identified rice *CCoAOMT* sequences. Total RNAs extracted with the TRIzol agent (Invitrogen) were used for reverse transcription with the ImProm-II™ Reverse Transcription System (Promega). The obtained cDNA were amplified by using their corresponding primers. The amplified products were cloned into pGEM-T Easy vector (Promega) and the clones were confirmed by sequencing.

(ⅲ) Amino acid sequence comparisons and phylogenetic tree construction. The deduced amino acid sequences of the three rice *CCoAOMT* genes were used for blast searches against the GenBank databases and nine *CCoAOMT* sequences from different plants were selected. The sequences are from *Eucalyptus gunnii* (004854), *Petroselinum crispum* (CAA90894), *Populus tomentosa* (AF240466), *Pinus taeda* (AAD02050), *Nicotiana tabacum* (004899, 024149), *Vitis vinifera* (043247) and *Zea mays* (CAB45149, CAB45150). The multiple sequence alignment was performed with the DNAman software. Phylogenetic analysis was made by PAUP 4.0 beta 10 win.

(iv) Northern blot analyses. Total RNA (20 μg) of roots, stems and leaves was separated on denatured formaldehyde gels, transferred onto nylon membrane and then hybridized with α-^{32}P-dCTP labeled cDNA. Probes were labeled by using the instruction RadPrime DNA Labeling System (Invitrogen). Hybridization, washing and detection were carried out as described by Sambrook et al.[10].

(v) *In situ* hybridization. The stems of 14-day-old rice were immersed in FAA fix solution overnight, dehydrated through a graded ethanol series, cleared in a series of alcohol and xylene, and then embedded in paraffin. Microtome sections (10 μm thick) were cut and mounted on polylysine-coated glass slides. RNA probe preparation was performed with T7/Sp6 DIG RNA Labeling Kit (Roche). Hybridization with a digoxygenin-labeled RNA probe and immunological detection were conducted as described by Xu et al.[11]. A bright field microscope was used to visualize the hybridization signals.

2 Results and discussion

(i) Gene structure. Three obtained rice *CCoAOMT* were designated as *OsCOA1*(accession number AY644636), *OsCOA20* (accession number AY644637) and *OsCOA26* (accession number AY 644638). The full-length of *OsCOA1* is 1072 bp and consists of four exons and three introns (Fig. 1). Its open reading frame (ORF) was 783 bp and encoded for a 260 amino acid polypeptide with a calculated molecular mass of 29 kD and predicted isoelectric point (*pI*) of 5.06. *OsCOA20* and *OsCOA26* were 958 and 916 bp in length, with their ORFs being 759 and 705 bp respectively. They have a similar gene structure, containing three exons and two introns. The predicted peptides contain 252 and 234 amino acid. residues, with estimated molecular masses of 28 and 26 kD, and calculated *pI* values of 4.94 and 5.48 for *OsCOA20* and *OsCOA26*, respectively. The GC content of the coding region of each gene ranges from 66% to 69%, which is typical for a graminaceous monocot since the monocot genes are highly biased toward codons ending in C or G[12].

(ii) Amino acid sequence comparisons and phylogenetic analysis. Sequence analyses showed that three rice *CCoAOMT* genes share a 60.23% identity and a 75.43% identity with their corresponding homologues in other plants. All regions were highly conservative except the 5' end (Fig. 2).*OsCOA1* contains all conserved motifs, i.e. motifs A, B, C, D, E, F, G, H, and shares a higher identity with other *CCoAOMT* genes. Motifs A, B and C are common in plant OMTs and were thought of as specific SAM-binding sites of which distances were similar to those of the previously studied *OMT* genes. The specific spatial arrangement might be essential for the formation of the SAM-binding pocket[4]. *OsCOA20* and *OsCOA26* have high degree of sequence similarity in motifs A, D, E, F and G, while motifs B, C and H are divergent. It remains to be investigated whether the divergence is concerned with the substrate specificity.

Phylogenetic analyses indicated that the sequences belong to two groups (Fig. 3). OsCOA1 and zea CCoAOMT were found to be in the same group, showing the closest relationship to the zea CCoAOMT proteins. This suggested that OsCOA1 might be a conserved sequence during monocot evolution. OsCOA20 and OsCOA26 form the other group, which might be specific in rice.

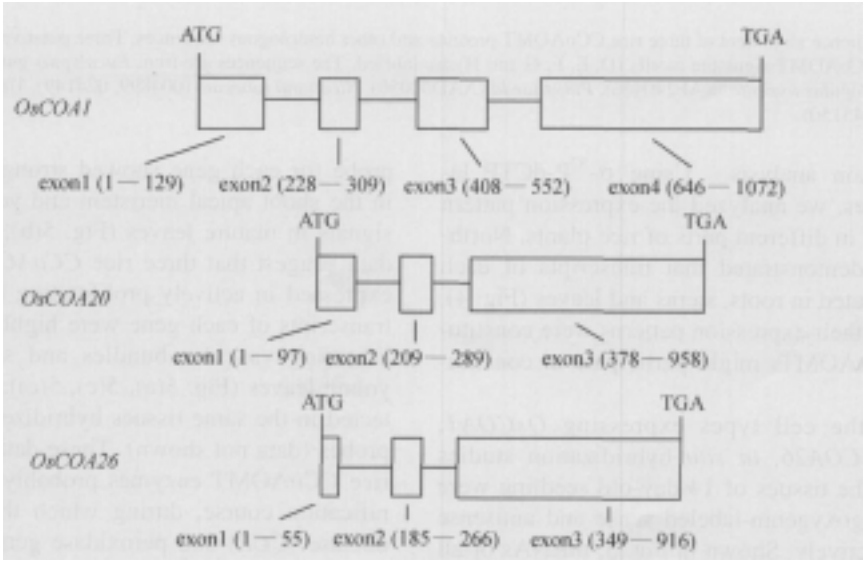

Fig. 1. The structure of three rice *CCoAOMT* genes.

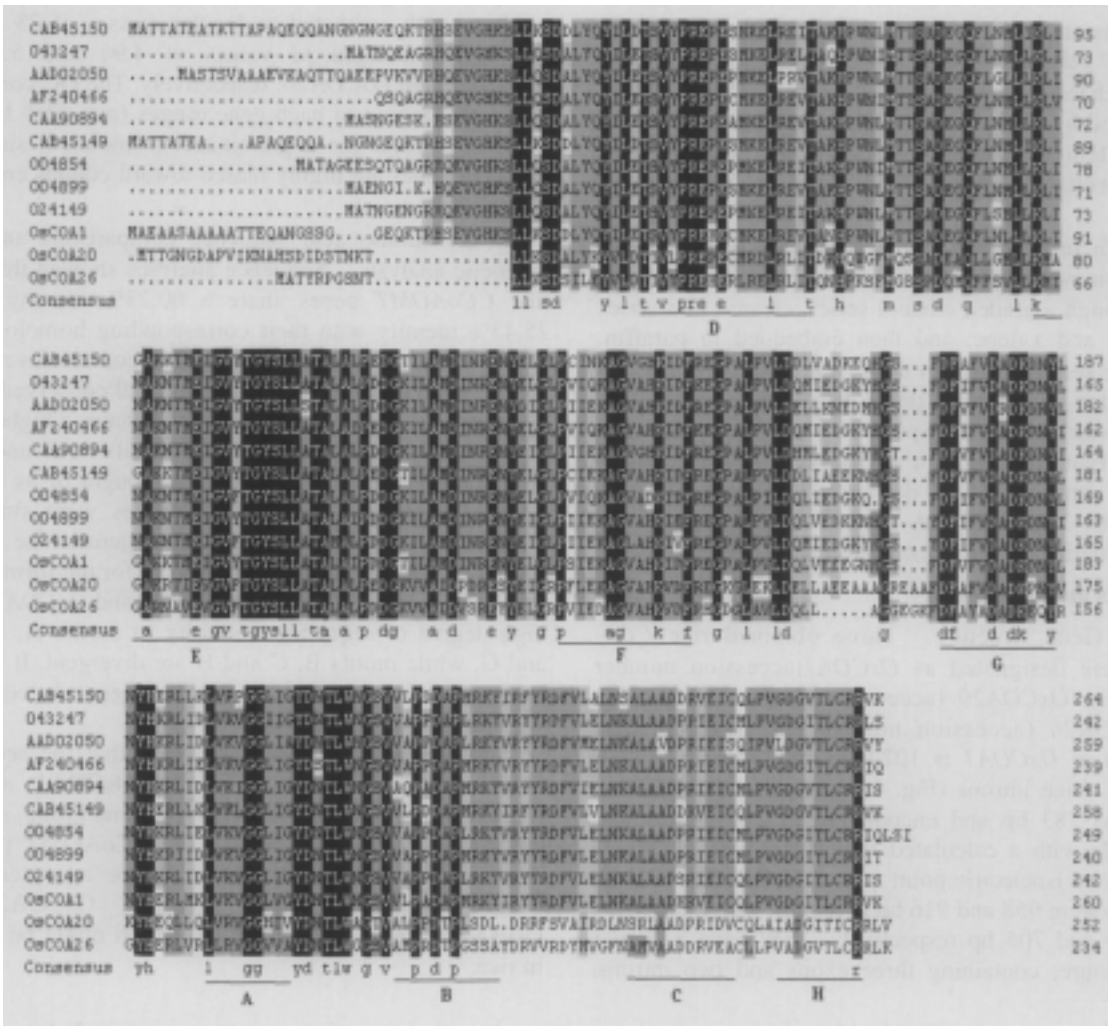

Fig. 2. Amino acid sequence alignment of three rice CCoAOMT proteins and other homologous sequences. Three putative SAM binding motifs (A, B and C) and additional CCoAOMT signature motifs (D, E, F, G and H) are labeled. The sequences are from *Eucalyptus gunnii* (004854), *Petroselinum crispum* (CAA90894), *Populus tomentosa*(AF240466), *Pinus taeda* (AAD02050), *Nicotiana tabacum* (004899, 024149), *Vitis vinifera*(043247) and *Zea mays* (CAB45149, CAB45150).

(iii) Expression analysis. Using α-^{32}P-dCTP labeled cDNAs probes, we analyzed the expression pattern of each *CCoAOMT* in different parts of rice plants. Northern blot analyses demonstrated that transcripts of each gene were accumulated in roots, stems and leaves (Fig. 4). This indicated that their expression patterns were constitutive and three CCoAOMTs might participate in constitutive lignification.

To localize the cell types expressing *OsCOA1*, *OsCOA20* and *OsCOA26*, *in situ* hybridization studies were performed. The tissues of 14-day-old seedling were hybridized with digoxygenin-labeled sense and antisense RNA probes respectively. Shown in Fig. 5, mRNAs of all three genes were accumulated in the shoot apex and lateral root meristem. In the shoot apex region, the antisense probe for each gene showed strong hybridization signals in the shoot apical meristem and young leaves, but weak signals in mature leaves (Fig. 5(b), 5(d) and 5(f)). These data suggest that three rice *CCoAOMT* genes are mainly expressed in actively proliferating tissues. Moreover, the transcripts of each gene were highly accumulated in proliferating vascular bundles and sclerenchyma cells in young leaves (Fig. 5(a), 5(c), 5(e)). Few signals were detected in the same tissues hybridized with the sense RNA probes (data not shown). These data suggest that all three rice CCoAOMT enzymes probably participate in the lignification course, during which the cinnamoyl-CoA reductase (*CCR*) and peroxidase genes are also highly expressed. Therefore, these genes might be responsible for the biosynthesis of phenolic compounds and lignin pre-

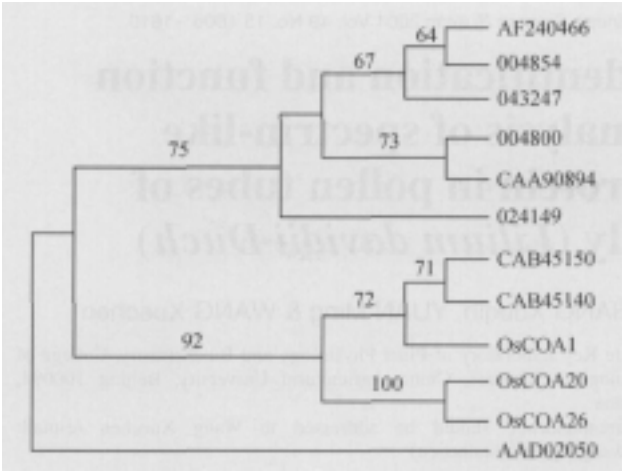

Fig. 3. Phylogenetic tree of rice CCoAOMT and other homologous sequences. The sequences are from *Eucalyptus gunnii* (004854), *Petroselinum crispum* (CAA90894), *Populus tomentosa*(AF240466), *Pinus taeda*(AAD02050), *Nicotiana tabacum* (004899, 024149), *Vitis vinifera*(043247), and *Zea mays* (CAB45149, CAB45150).

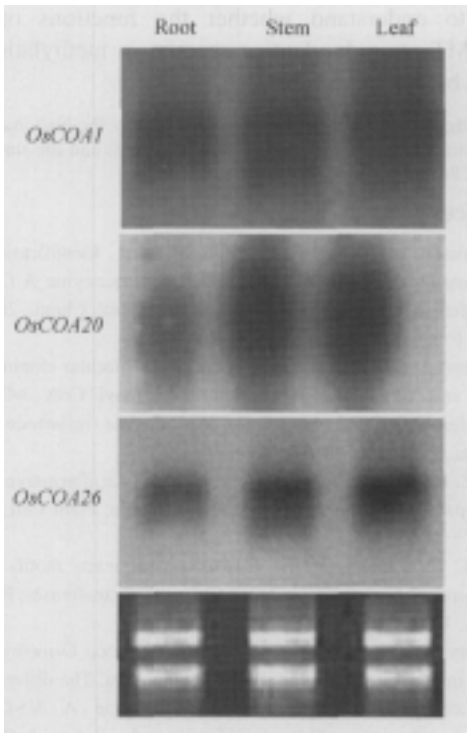

Fig. 4. Northern blot analyses of three rice *CCoAOMT* genes.

cursors in proliferating tissues[13,14].

Our studies demonstrated that three rice *CCoAOMT* genes were closely related with lignification, suggesting the utilization of these genes in altering lignin biosynthesis via transgenic research. Interestingly, the expression of each gene took on a similar pattern, implying that these three CCoAOMT enzymes may possibly participate in lignin biosynthesis pathway altogether. However, sequence analyses demonstrated that the gene structures of *OsCOA20* and *OsCOA26* showed differences from that of *OsCOA1*. Moreover, some conserved motifs concerned with SAM- or substrates-binding sites in OsCOA20 and OsCOA26 were more divergent than those of the CCoAOMT from other plants. It suggests that they might have distinct substrate specificities or have specific functions in specific stage during lignification course. Therefore the substrate preferences of each enzyme and their specific functions in plant development and defense mechanism need to be investigated in detail. It will be

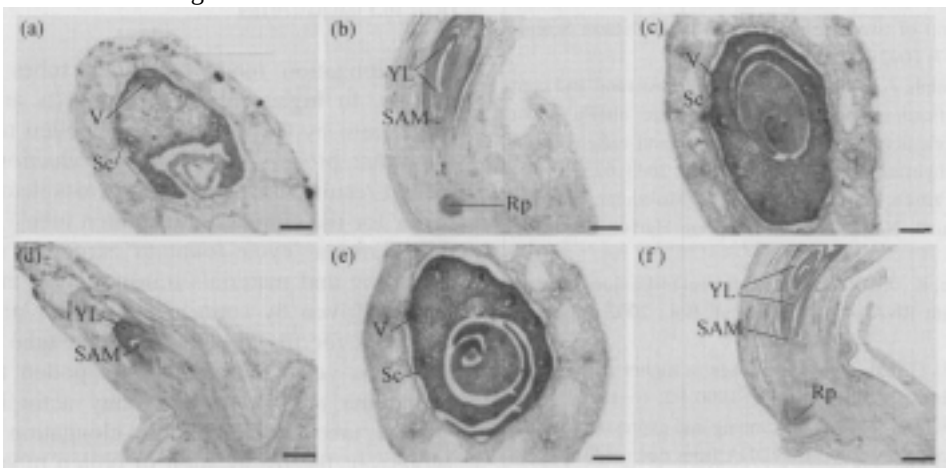

Fig. 5. *In situ* hybridization analysis of three *CCoAOMT* in rice. Stems of 14-day-old rice were used for *in situ* hybridization analysis and hybridized with digoxygenin-labeled antisense *OsCOA1* ((a) and (b)), *OsCOA20* ((c) and (d)) and *OsCOA26* ((e) and (f)). Panels (a), (c) and (e) show the transverse sections of leaves and sheaths, bars = 100 μm, panels (b), (d) and (f) represent the longitudinal stem sections, bars = 400 μm. SAM, shoot apical meristem; Sc, sclerenchyma cells; Rp, branch root primordia; V, vascular bundles; YL, young leaves.

helpful to understand whether the functions of three CCoAOMT are redundant or specific in methylation step of lignin biosynthesis.

Acknowledgements This work was supported by the National Natural Science Foundation of China (Grant No. 30170783) and the state " 863" High-Tech Program (Grant No. 2004AA212121).

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(Received May 24, 2004; accepted June 28, 2004)

Chinese Science Bulletin 2004 Vol. 49 No. 15 1606—1610

Identification and function analysis of spectrin-like protein in pollen tubes of lily (Lilium davidii Duch)

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Abstract The elongation of pollen tube is an important process of sexual reproduction in higher plant. Cytoskeleton plays a major regulatory role in the elongation of pollen tubes. But whether membrane skeleton is involved in the pollen tube elongation is not clear. In this study, imunochemical detection of spectrin-like protein has been carried out in pollen tubes. By use of 2-dimensional electrophoresis(2DE) and western blotting, two spectrin-like proteins are found, one is 150 kD, and the other is 105 kD, with pI being 4.54 and 4.39, respectively. 150 kD spectrin-like protein is located in plasma membrane of pollen tube and 105 kD spectrin-like protein is located in cytoplasm, probably functioning as a subunit to form a dimmer (210 kD) in vivo. The elongation of pollen tubes is inhibited after spectrin antibody was injected into a growing pollen tube. These results suggest that spectrin-like proteins exist in pollen tube and play an important regulating role in the elongation process of pollen tubes from lily.

Keywords: spectrin, microfilament, actin-binding-proteins, pollen tubes.
DOI 10.1360/04wc0169

Elongation mode of pollen tubes in plant is tip growth. In higher plant, sperm cells are transferred to megaspore by the elongation of pollen tubes. So it is an important process of sexual reproduction. Many experimental results show that actin cytoskeleton is an essential factor for the elongation of pollen tube[1]. Actin in pollen tube forms a "cycle fountain" structure, and cytoplasmic streaming and materials transportation in pollen tube are mainly driven by actin and its motor proteins. Essential materials for tip growth of pollen tube are often transported as vesicles and fused at pollen tip to form new membrane and cell wall. Many actin binding proteins (ABPs) are involved in the elongation of pollen tube. Moreover, the tip location of pollen tube is composed of highly dynamic short actin bundles, and the dynamics of these short actin bundles is important for tip growth of pollen tube[2]. Actin-bound proteins are involved in the dynamics of actin cytoskeleton[3]. In addition, vesicles transportation and membrane fusion occur at the tip of