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cDNA Cloning and Sequence Analysis of the Rice Cinnamate-4-Hydroxylase Gene, a Cytochrome P450-Dependent Monooxygenase Involved in the General Phenylpropanoid Pathway

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Plant cytochrome P450 monooxygenases (P450s) mediate a wide range of oxidative reactions involved in the biosynthesis of phenylpropanoids, terpenes, lipids, and alkaloids. We isolated a cDNA clone for cinnamate-4-hydroxylase (C4H) from a Japonica type rice (*Oryza sativa* L. cv. Ilpumbyeo). This C4H has a deduced amino acid sequence that is 85% identical to *that* of *Sorghum bicolor*. Our phylogenetic analysis also showed that the *OsC4HL* gene is closely related to *C4H* from *S. bicolor*. A putative genomic DNA sequence corresponding to *OsC4HL* contained *cis*-elements (boxes P, A, L, and TCA motifs), AT-rich elements, and wound-response elements that control gene expression in its promoter region. *OsC4HL* expression was detected in all the tissue types, with the highest level being measured in the roots. It was also apparently up-regulated by wounding stress. These data suggest that the *OsC4HL* gene is *C4H* member in the *CYP73* subfamily.

Keywords: C4H, OsC4HL, rice

The superfamily of cytochrome P450-dependent monooxygenases (Cyt P450s) is a large group of heme-containing enzymes that catalyze NADPH- and O_2-dependent hydroxylation reactions (Chapple, 1998; Whitbred and Schuler, 2000). Cyt P450s have been found in bacteria, insects, fish, mammals, plants, and fungi. Among these, plant Cyt P450s participate in many biochemical pathways, such as for phenylpropanoids, alkaloids, terpenoids, lipids, cyanogenic glycosides, glucosinolates, and brassinosteroids (Gravot et al., 1993; Frank et al., 1996; Urban et al., 1997; Ro et al., 2001).

Cyt P450 genes that function in the phenylpropanoid pathway have been isolated and characterized in several plants species. The importance of this pathway is demonstrated by the variety of secondary metabolites involved in the differentiation and protection of plant tissues against environmental stresses (Hahlbrock and Scheel, 1989; Gravot et al., 1993; Mizutani et al., 1997). For example, cinnamate-4-hydroxylase (C4H), phenylalanine ammonia lyase (PAL), and 4-coumarate CoA ligase (4CL) are key enzymes in the core phenylpropanoid pathway. They provide direct carbon flux to an array of critical phenolic compounds, e.g., lignin, suberin, flavonoids, and numerous other phenylpropanoids (Chapple, 1998). These compounds are also controlled by other genes for flavonoids (*CHS*), for lignin (*C3H, F5H, OMT, pCCoA 3H, CCoA OMT*, and *SINAPOYL CoA*), and for sinapic acid esters (*C3H, F5H, OMT, SGT, SCT*, and *SMT*) (Frank et al., 1996; Mizutani et al., 1997; Ruegger et al., 1999; Overkamp et al., 2000).

Among these, C4H has been the most extensively studied because it gives rise to numerous important metabolites, including lignin, flavonoids, and hydroxycinnamic acid esters. This enzyme, the second in the phenylpropanoid pathway, catalyzes the *para*-hydroxylation of *trans*-cinnamic acid derived from *phenylalanine* by the action of PAL (Fig. 1). Likewise, it defines the *CYP73* subfamily in the Cyt P450 superfamily (Nelson et al., 1993; Chapple, 1998; Ruegger et al., 1999). Moreover, C4H controls the carbon flux for numerous phytoalexins that are synthesized when plants are challenged by pathogens (Teutsch et al., 1993). Expression of the *C4H* gene is also induced by wounding and UV-irradiation, and is controlled by *cis*-acting elements in the promoter region (Teutsch et

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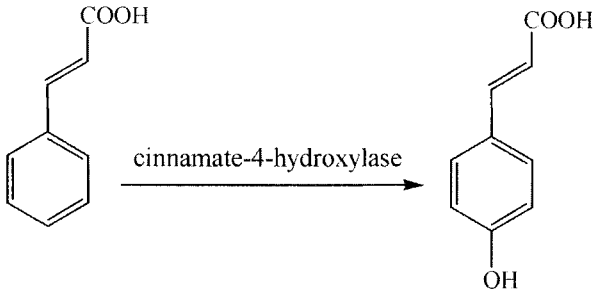

Figure 1. Conversion of *trans*-cinnamic acid to *p*-coumaric acid is catalyzed by cinnamate-4-hydroxylase.

al., 1993; Bell-Lelong et al., 1997; Mizutani et al., 1997).

Several *C4H* cDNAs and genomic clones have been reported from many dicot species, e.g., *Agastache rugosa*, *Arabidopsis thaliana*, *Camptotheca acuminata*, *Catharanthus roseus*, *Capsicum annuum*, *Capsicum chinense*, *Cicer arietinum*, *Glycine max*, *Glycyrrhiza echinata*, *Helianthus tuberosus*, *Lithospermum erythrorhizon*, *Medicago sativa*, *Petroselinum crispum*, *Pinus taeda*, *Pisum sativum*, *Populus kitakamiensis*, *Populus tremuloides*, *Ruta graveolens*, *Vigna radiate*, and *Zinnia elegans*. In monocots, *C4H* has been reported only from *Sorghum bicolor*. Rice is not only an important food crop, but also serves as a scientific model system for monocots due to its small genome, high synteny to other monocot species, and great efficiency in its transformation (Ahn and Tanksley, 1993; Hiei et al., 1994; Havukkala, 1996; Izawa and Shimamoto, 1996). However no genetic information about a rice *C4H* has been reported, although *PAL* and *4CL* have been isolated from that genus (Zhao et al., 1990; Zhu et al., 1995). Therefore, our objective here was to isolate and characterize rice *C4H*, analyze the sequence of its putative promoter region, and describe its expression patterns in various tissue types and in response to wounding stress.

MATERIALS AND METHODS

Plant Material and Wounding Treatment

Rice (*Oryza sativa* L. cv. Ilpoombyeo) seedlings were hydroponically cultivated in a half-strength Murashige and Skoog (MS) nutrient solution. The growth chamber was maintained at 28/20°C (D/N), under a 14-h photoperiod. Photosynthetic photon flux density (PPFD) at pot level was 330 μmol m^{-2} s^{-1}, supplied from two natrium lamps and six fluorescent lamps. For the extraction of total RNA and genomic DNA, seedlings were harvested and divided into roots, stems, and leaf portions at 3 to 10 weeks after sowing. The tissues were immediately frozen in liquid nitrogen and stored at -70°C.

For the wounding treatment, 2/5 (W/L) fragments were excised 1 cm above the stem (node) base of 10-week-old seedlings. The wounded plants were then incubated for 1 to 9 h under the previously described growing conditions. Afterward, 2-cm-long stem fragments, including the wounded section, were collected and frozen in liquid nitrogen.

Reverse Transcription (RT)-PCR Analysis and Rapid Amplification of cDNA Ends (RACE)

Total RNA was isolated with Trizol reagent (Invitrogen, USA), according to the manufacturer's protocol. For RT-PCR analysis, 5 μg of total RNA was reverse-transcribed using the RevertAid™ H Minus First Strand cDNA Synthesis Kit (MBI Fermentas, USA). Products of the first-strand cDNA synthesis reaction were amplified by PCR using *OsC4HL* primers forward (5′-CGACCACTGGCGAAAGATGCGGCGG-3′) and reverse (5′-AGCAGCTCGAAGTTCTGGACGAGGC-3′), which were designed by comparing the sequences of *C4H* genes from *A. rugosa* (GenBank AY616436), *C. acuminata* (AY621152), *C. annuum* (AF212318), *L. erythrorhizon* (AB055508), *P. kitakamiensis* (D82812), *R. graveolens* (AF548370), and *S. bicolor* (AY034143). Partial cDNAs were isolated using the method of 5′ rapid amplification of cDNA ends (RACE) (Frohman et al., 1988). For 5′ RACE, oligo(dT)-primed cDNA was tailed and amplified with a 5′/3′-RACE kit (Roche Diagnostics, USA) and four additional *OsC4HL*-specific primers, including c41sp1 (5′-CACCGTAGTTGTACTCGAAGC-3′), c41sp2 (5′-CGGAACATGTCGTTGTACATCAT-3′), c41sp3 (5′-CCACCACCTTGTTGGTGAAGA-3′), and c42sp1 (5′-CTCTCCAAGGACCTCCTCCT-3′). The amplified fragments were cloned into the pGEM-T-easy plasmid vector (Promega, USA) and sequenced.

cDNA, Genomic DNA Sequence, and Phylogenetic Analyses

The sequence of *OsC4HL* was analyzed online at the following websites: http://www.ncbi.nlm.nih.gov, http://www.shigen.nig.ac.jp, http://www.gramene.org, and http://www.softberry.com. Amino acid sequences were aligned by Clustal X (Thompson et al., 1997), and phylogenetic trees were generated by the neighbor joining

method with 1000 repeats, using MEGA2 (Kumar et al., 2001). All neighbor-joining trees were drawn by NJplot (Perrière and Gouy, 1996). Gene designations and their GenBank accession numbers used for sequence comparisons included: C4H (AY616436; *A. rugosa*), C4H (AY621152; *C. acuminata*), C4H (AF548370; *R. graveolens*), C4H-2 (AB055508; *L. erythrorhizon*), C4H-1 (AB055507; *L. erythrorhizon*), C4H (AY034143; *S. bicolor*), C4H (AF212318; *C. annuum*), CYP73 (X92437; *G. max*), cyp73a (D82812; *P. kitakamiensis*), CYP Ge-1 (D87520; *G. echinata*), CYP73A12 (U19922; *Z. elegans*), CYP73A3 (L11046; *M. sativa*), CYP73A1 (Q04468; *H. tuberosus*), CYP73A5 (P92994; *A. thaliana*), CYP73A19 (O81928; *C. arietinum*), CYP73A13 (O24312; *P. tremuloides*), C4H (P37115; *V. radiata*), CYP73A10 (L38898; *P. crispum*), CYP73A4 (Z32563; *C. roseus*), CYP73A9 (AF175275; *P. sativum*), TC4H (AF096998; *P. taeda*), and Ca4h (AF088847; *C. chinense*).

Southern Blot Analysis

Genomic DNA was isolated from young rice leaves using the DNeasy Plant Mini Kit (Qiagen, USA). Aliquots (10 μg) of genomic DNA were digested with *Eco*RV, *Bam*HI, or *Hind*III, then fractionated on 0.8% (w/v) agarose gels. Each gel was blotted onto a nylon membrane (Hybond N^+; Amersham, UK), which was then crosslinked. The membranes were hybridized with ^{32}P-labeled cDNA fragments that included the 0.6-kb *OsC4HL* open reading frame (ORF) as a probe. Hybridization was carried out at 65°C overnight in a modified Church buffer (Church and Gilbert, 1984) that contained 7% sodium dodecyl sulfate (SDS), 0.5 M EDTA, 0.5 M sodium phosphate, and 1% bovine serum albumin. The blots were washed twice with 2× SSC, 0.1% SDS for 20 min at 65°C; then twice with 1× SSC, 0.1% SDS for 10 min at 65°C; and finally exposed for 1 d using the PERSONAL MOLECULAR IMAGER FX system (Bio-Rad, USA) for 1 d.

Northern Blot Analysis

Total RNA was isolated as described above. Samples were electrophoretically separated on 1% agarose gels containing 1X MOPS solution and 5% formaldehyde, then transferred to Hybond N^+ nylon membranes. These were hybridized with ^{32}P-labeled cDNA fragments that included the 0.6-kb *OsC4HL* ORF as a probe. Hybridization was carried out at 65°C overnight in a modified Church buffer that contained 7% SDS, 0.5 M EDTA, 0.5 M sodium phosphate and 1% (w/v) bovine serum albumin. The blots were washed twice with 2× SSC, 0.1% SDS for 20 min at 65°C; then twice with 1× SSC, 0.1% SDS for 10 min at 65°C; and finally exposed for 2d using the PERSONAL MOLECULAR IMAGER FX system.

RESULTS AND DISCUSSION

Isolation and Sequence Analysis of cDNA Clone Encoding *OsC4HL* Gene from Rice

We isolated cDNA clones from rice via RT-PCR and the 5′/3′ RACE method. Sequences analysis of the *OsC4HL* cDNA showed that it has an open reading frame of 1503 b and encodes a putative protein of 501 amino acids. BLAST searches revealed that one of these clones shares high sequence identities of 85% and 73% with *C4H* of *S. bicolor* and *Citrus sinensis*, respectively. Because these results suggest that the isolated cDNA clone represents an *OsC4H*-like gene from *O. sativa* L., we have named this gene *OsC4HL* (*O. sativa* L. *C4H*-like gene). Its predicted amino acid sequences exhibit the primary characteristics of Cyt P450s, which possess a heme-binding region (FxxGxxxCxG) near the C terminus and a consensus sequence (PPGPx(G/P)xP) of the proline hinge in the N terminal region (Fig. 2).

We conducted a phylogenetic analysis of known *C4H* (*CYP73*) genes using most of the published genes that belong to the *CYP73* subfamily (Nelson, 1993). Our analysis showed that this gene falls into that subfamily as well (Fig. 3). *OsC4HL* also shows high affinity with monocot *C4H* genes from *S. bicolor.*

Southern blot analysis was performed to estimate the number of *OsC4HL* genes in the entire rice genome. Genomic DNA was digested with three restriction enzymes -- *Bam*HI, *Eco*RV, and *Hind*III -- that do not cut within the *OsC4HL* cDNA. As a probe for blotting, we used a cDNA fragment of the *OsC4HL* gene that excluded the highly conserved heme-binding and proline hinge regions was used. Only one band was ovserved in each lane, which implies that *OsC4HL* exists as a single copy in the rice genome (Fig. 4).

Analyses of Rice C4H Genomic Sequence and Its Promoter Region

When its genomic sequences were estimated, we found that *OsC4HL* occurs on Chromosome 5 of

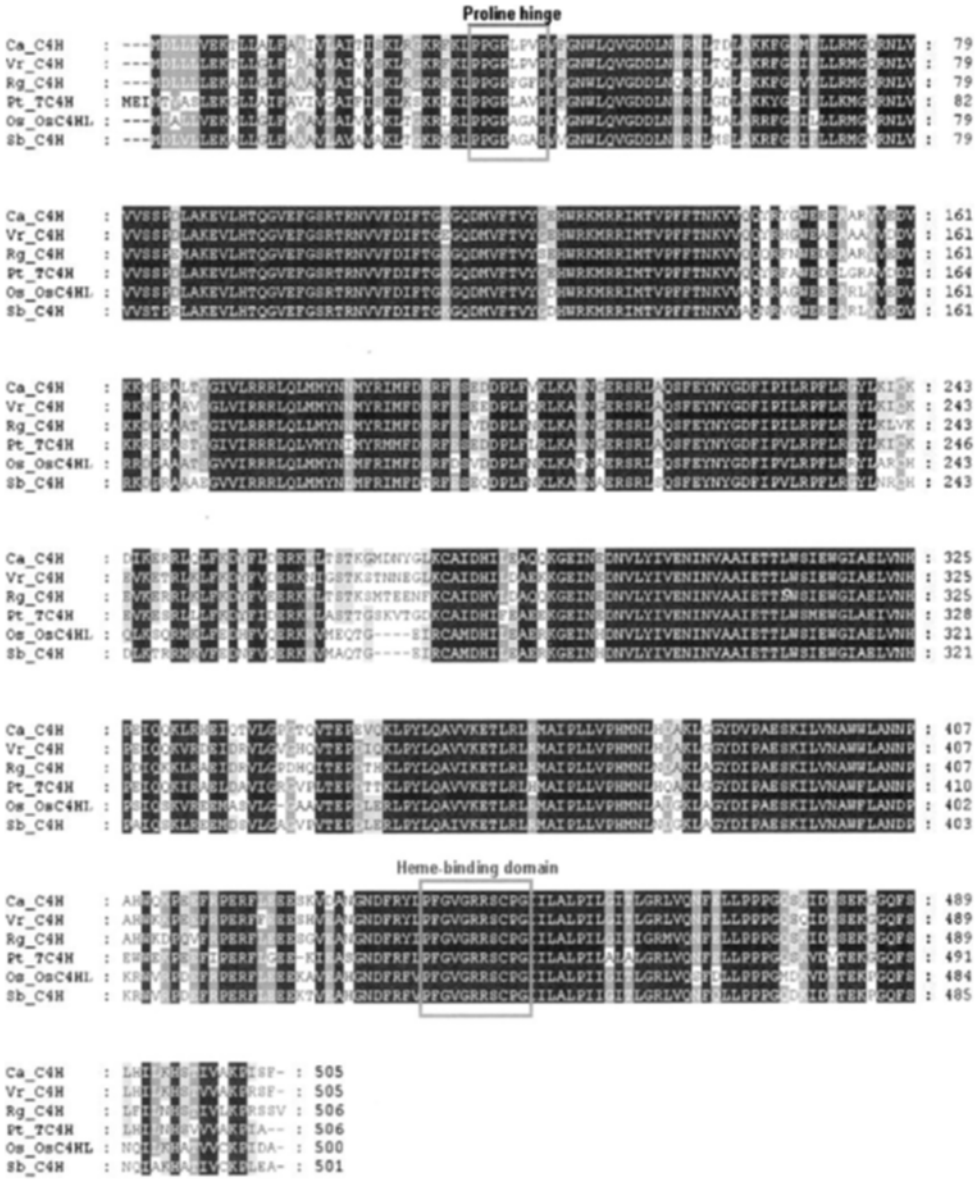

Figure 2. Alignment of the amino acid sequences for *C4H* genes: Ca_C4H, *C. acuminata*; Vr_C4H, *V. radiata*; Rg_C4H, *R. graveolens*; Pt_TC4H, *P. taeda*; Sb_C4H, *S. bicolor*. Identical residues are displayed in reverse type; similar residues are in gray boxes. Proline hinge and heme-binding domains are indicated by rectangles.

the rice genome, its coding sequence being interrupted by two introns (Intron I, 1640 b; Intron II, 1089 b).

We analyzed a putative promoter region responsible for the expression of *C4H*. The deduced transcription start site for *OsC4HL* is located 73 b upstream of the initiator ATG. A putative TATA box is found 34 b upstream from the start site, a putative CAAT at -194, and a CCAAA box at -59. The promoter region also contains *cis*-acting elements (box P, CCAa/cCa/tCC; box A, CCGTCC; box L, a/tCTa/cACCTAa/cCa/c; TCA motifs, TCAa/ta/cTTCa/Tt); box H (CCTACC (N_7)CT); box G (CACGTG); AT-rich elements (TAATTAAT); and wound-response elements (WRE). These elements are involved in the light-, wounding-, and elicitor-inducibilities of *C4H* promoters (Frank et al., 1996; Koopmann et al., 1999; Whitbred and Schuler, 2000). The P and L boxes are associated with increased levels of C4H in UV light-treated parsley cells and in fungal elicitor-treated cells (Bell-Lelong et al., 1997; Mizutani et al., 1997), while the H and G boxes are responsible for induction of *C4H* expression in fungal elicitor-

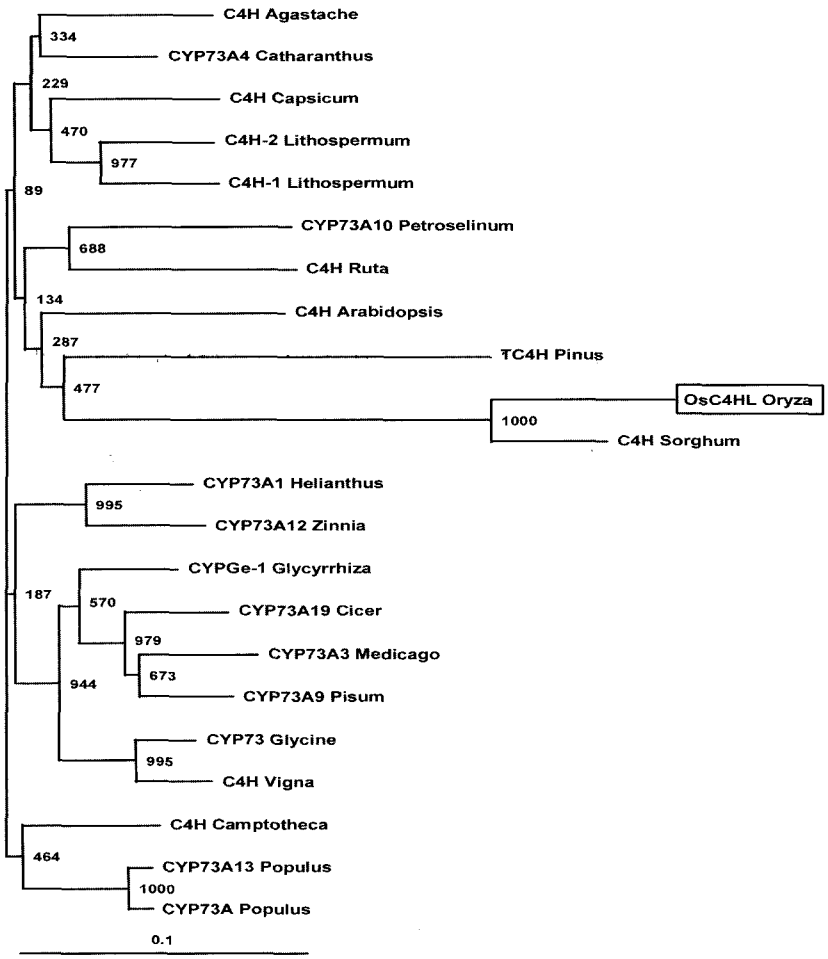

Figure 3. Phylogenetic relationship among *C4H* genes belonging to *CYP73* (*C4H*) subfamily. Neighbor-joining tree was generated by Clustal X. Numbers next to nodes indicate bootstrap values from 1,000 replicates. *OsC4HL* gene is boxed.

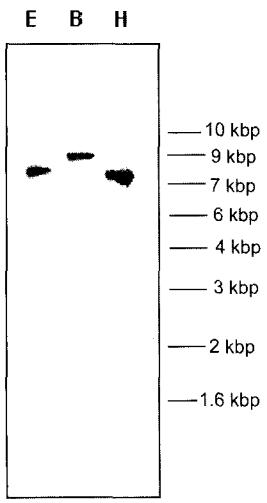

Figure 4. Southern blot analysis of *OsC4HL* in rice. Genomic DNA (10 μg per lane) was digested with enzymes, then hybridized with approx. 0.6-kbp cDNA fragment of *OsC4HL* as probe. E, *Eco*RV; B, *Bam*HI; H, *Hind*III.

treated cells. The G box sequence has also been identified in light-inducible promoters (Whitbred and Schuler, 2000). In addition, TCA motifs, found at -419 b and -1137 b of the *OsC4HL* promoter, are characteristic of stress-responsive promoters (Goldsbrough et al., 1993; Kato et al., 1995). Whitbred and Schuler (2000) have reported three wound-response elements -- WRE 1 (AAATTC), WRE2 (AT-rich), and WRE3 (CCACCT) -- in the promoter region of *C4H*. In particular, the AT-rich (WRE2) element has been identified as being responsible for the elicitor-induced expression of *C4H*. Furthermore, the A and P boxes belonging to *cis*-elements share a consensus sequence with the AC and WRE3 elements, respectively (Fig. 5). This result implies that the elements in the promoter region interact in controlling *C4H* expression in response to wounding, elicitors, or treatments with UV light (Whitbred and Schuler, 2000).

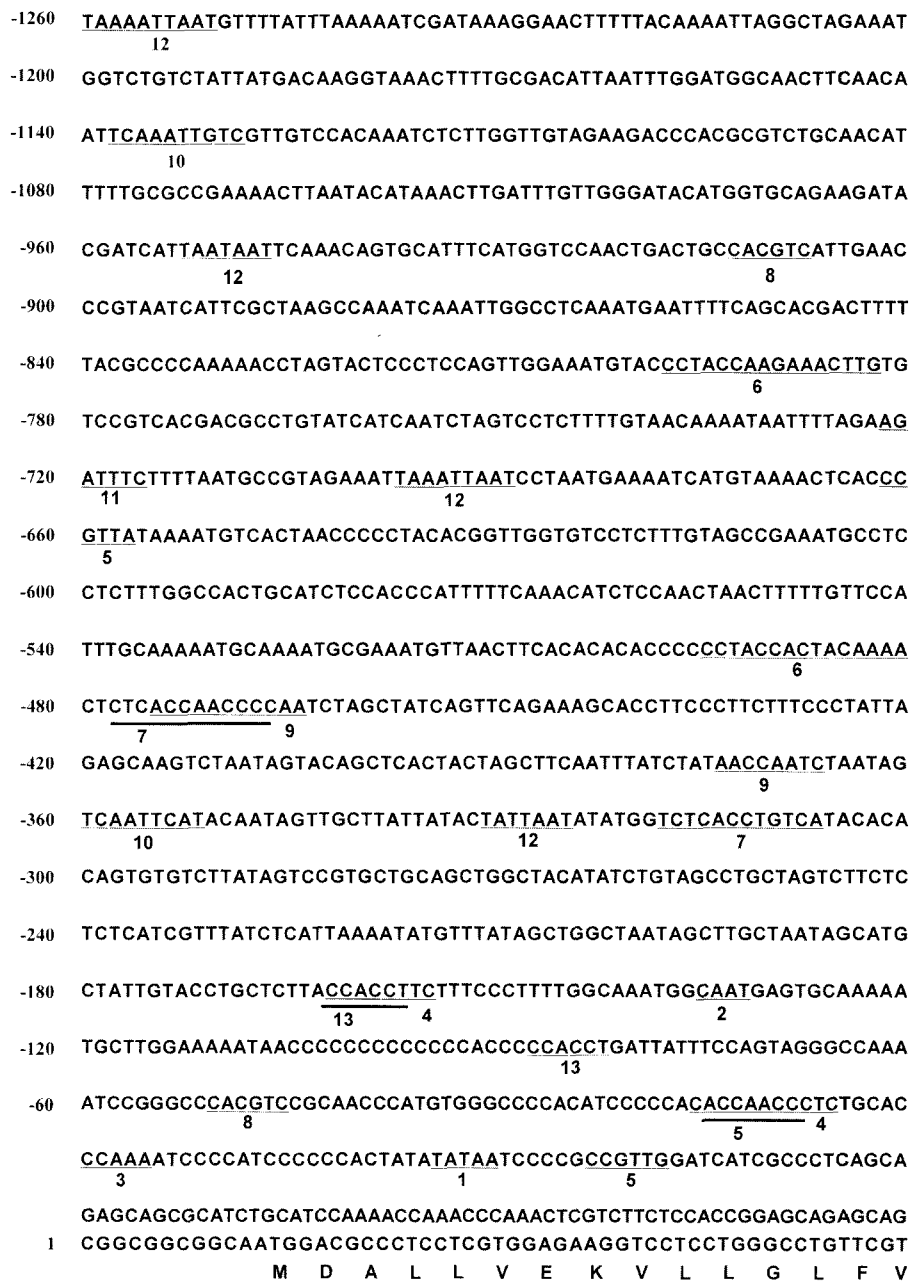

Figure 5. Putative promoter region in genomic DNA sequence of *OsC4HL* gene. Numbers corresponding to nucleotide sequence are on right side. 1, TATAA box; 2, CAAT box; 3, CAAA sequence; 4, P box; 5, A box; 6, H box; 7, L box; 8, G box; 9, AC element; 10, TCA motif; 11, WRE1; 12, AT-rich element (WRE2); 13, WRE3.

C4H Expression in Rice

The expression patterns of *OsC4HL* were investigated using total RNA isolated from roots, stems, and young and mature leaves (Fig. 6). Northern blot analysis revealed that *OsC4HL* transcripts were detected in all tissue types, but showed their highest level in roots; amounts were also relatively higher in the levels than in the stems. Other researchers have also reported that *C4H* promoter-driven GUS activity is highly detectable in the roots of *Arabidopsis* (Ohl et al., 1990; Hatton et al., 1995; Lee et al., 1995). This differential expression of *C4H* would depend on the activation state of its promoter by transcription factors, e.g., *AtMYB4*, which acts as a strong negative regulator for *C4H* but not for other phenylpropanoid

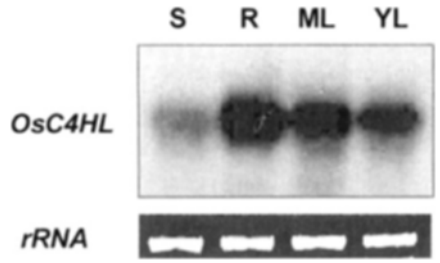

Figure 6. Northern blot analysis of *OsC4HL* expression in different rice tissues. Total RNA (10 μg per lane) was loaded onto gel; equal loading was verified by ethidium bromide-staining of 28S ribosomal RNA bands. S, stems; R, roots; ML, mature leaves; YL, young leaves.

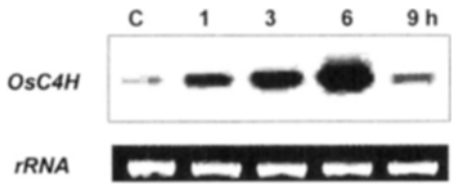

Figure 7. *OsC4HL* expression in rice after wounding. Total RNA was isolated from stems before treatment (control, C), or at 1, 3, 6, or 9 h afterward. Total RNA (10 μg per lane) was loaded onto a gel; equal loading was verified by ethidium bromide-staining of 28S ribosomal RNA bands.

pathway genes such as *PAL* and *4CL* (Ro et al., 2001; Anterola et al., 2002).

Because up-regulation of *C4H* is rapidly induced by light, wounding, elicitors, or pathogen infection (Mizutani et al., 1997; Koopmann et al., 1999; Whitbred and Schuler, 2000; Ro et al., 2001), we investigated the effect of wounding on *OsC4HL* expression. This treatment gives rise to rapid and high production of lignin precursors and defense compounds (Bell-Lelong et al., 1997; Mizutani et al., 1997). Likewise, we found here that *OsC4HL* transcript levels were significantly increased within 1 h of wounding (Fig. 7). Expression reached a maximum within 6 h after treatment, but then decreased, probably due to the saturation of lignin precursors synthesized.

In conclusion, *OsC4HL* contains 13 elements in its promoter region that are conserved in most *C4H* genes. Expression was highest in roots, and could be increased in response to wounding stress. These data demonstrate that the *OsC4HL* gene encodes cinnamate-4-hydroxylase in the rice *CYP73* subfamily.

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