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Analysis of five rice 4-coumarate:coenzyme A ligase enzyme activity and stress response for potential roles in lignin and flavonoid biosynthesis in rice

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ABSTRACT

4-Coumarate:coenzyme A ligase (4CL) catalyzes the conversion of hydroxycinnamates into corresponding CoA esters for biosynthesis of flavonoids and lignin. In this study, five members of the 4CL gene family from rice were cloned and analyzed. Recombinant 4CL data revealed that 4-coumaric acid and ferulic acid were the two main substrates of 4CL (Os4CL1/3/4/5) for monolignol biosynthesis in rice. Os4CL2 was specifically expressed in the anther and was strongly activated by UV irradiation, suggesting its potential involvement in flavonoid formation. Moreover, bioinformatics analysis showed that the existence of valine residue at the substrate-binding pocket may mainly affect rice 4CL activities toward sinapic acid.

1. Introduction

4-Coumarate:coenzyme A ligase (4CL) converts 4-coumaric acid (PA) and other substituted cinnamic acids, such as caffeic (CA), ferulic (FA), and sinapic acids (SA), into corresponding CoA thiolesters used for the biosynthesis of flavonoids, isoflavonoids, lignin, suberins, coumarins, and wall-bound phenolics [1,2]. 4CL genes have been cloned from numerous plant species where they exist in a small gene family, with two to five members [1,3–10]. The catalytic properties of 4CL isoforms have been studied extensively [1,3–6,8]. In some species, such as potato, parsley, and loblolly pine, the cloned genes encode identical or nearly identical proteins that possess similar substrate affinities [7,11,12]. In other plants, such as aspen and Arabidopsis, the 4CL family has structurally and functionally distinct members [1,4,8]. These isoforms control the relative abundance of flavonoids and various monolignols during normal development [13].

Phenylpropanoid metabolism is regulated primarily via transcriptional control of the corresponding genes. The accumulation of 4CL mRNAs and the activity of their promoters vary during tissue and cell differentiation when certain cells become specialized for the biosynthesis of phenylpropanoid-derived compounds, such as lignin, flavonoids, and anthocyanin pigment [14,15]. Gene expression is also induced upon environmental stresses, such as wounding, pathogen attack, and UV light irradiation, against which, phenylpropanoid compounds may have protective roles [16]. Thus, divergent 4CL isoforms have diverse functions in development and/or environmental stress responses.

4CLs are encoded by five genes in rice; their corresponding enzymatic properties have been characterized [3]. However, little is known about the nature of 4CL isoforms in directing metabolic flux for the biosynthesis of different classes of phenolics with specialized functions in rice. This study reports on the characterization of five 4CL isoforms in rice. Biochemical and *in vivo* expression, as well as phylogenetic analyses showed that Os4CL2 is associated with flavonoid biosynthesis, whereas the rest of the 4CLs (Os4CL1/3/4/5) participate in lignin formation. PA and FA are the two main substrates of 4CL for monolignol biosynthesis in rice.

2. Materials and methods

2.1. Plant material and stress treatments

Rice (*Oryza japonica*) seedlings were grown under short-day conditions (12 h light/28 °C, 12 h dark/22 °C) in chambers. For the wounding experiments, the seedlings were grown under short-day conditions for 2 weeks. Healthy looking, fully expanded leaves were detached, cut into 3 mm to 5 mm strips, placed on filter paper moistened with MS media in sealed Petri dishes, and

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incubated in the light. Samples were frozen in liquid nitrogen at indicated time points. Control leaves were detached and incubated without further wounding. For UV-light treatment, rice seedlings were grown under short-day conditions for 3 weeks. One warm white tube (T8-20W, Yaohu, China) and two UVA fluorescent tubes (UVA30W, Purple, China) were used for the illumination of the plants with UV-containing white light at 28 °C. Plants were harvested at different time points after the onset of irradiation and stored at −80 °C.

2.2. Expression in Escherichia coli and purification of Os4CL proteins

The cDNA from Nipponbare spikelets at the booting stage was used as template for the PCR amplification of five full-length 4CL cDNAs. The primer combinations for each gene are listed in Supplementary Table S1. All five Os4CL cDNAs were expressed in *E. coli* (BL21) using the expression vector, pGEX-6P-3. Recombinant protein expression, purification, and GST removal tag by enzymatic cleavage were carried out according to a previous method [17].

2.3. Enzyme assays

4CL activity was measured at 30 °C using a spectrophotometric assay to detect the formation of CoA esters of various cinnamic acid derivatives [1,13]. Kinetic constants (Km and Vmax) were estimated by linear regression of v/s against s (Hanes plot). Protein concentrations were determined according to Bradford, with bovine serum albumin as standard [18]. Three-substrate assays, containing 50 μM each of PA, CA, and FA, were conducted using the recombinant proteins (Os4CL1, 5 μg; Os4CL2, 1 mg; Os4CL3, 10 μg; Os4CL4, 2 μg; or Os4CL5, 1 μg). All assays were conducted in triplicate.

2.4. HPLC-UV analysis

HPLC-UV analysis of 4CL products was performed using an Agilent 1260 system. Up to 40 μL of the reaction mixture was loaded onto a C18 Discovery column (2.1 mm × 25 cm × 5 μm). The method for HPLC separation is listed in Supplementary Table S2. The thioesters were detected by UV absorbance at 340 nm. The reaction products were identified and quantified based on authentic standards.

2.5. Gene expression analysis

Total RNA was extracted from various tissues at different growth stages using the Trizol method based on the instructions of the manufacturer (Invitrogen). cDNA synthesis and semi-quantitative RT-PCR were performed as described by Wang et al. [19]. Q-PCR was carried out as described by Costa et al. [20]. PCR primers specific to each of the 4CL genes from rice are shown in Supplementary Table S3. Gene expression data were normalized using the rice ubq1 gene (Os06g0681400) as a reference. All experiments were conducted in triplicate.

2.6. Phylogenetic analysis

4CL amino acid sequences for the phylogenetic reconstruction were retrieved from GenBank. The accession numbers of these sequences are provided in Supplementary Table S4. The 4CL protein-coding sequences were aligned using the CLUSTALW implemented in MEGA5, and then manually edited. Neighbor-joining (NJ) analyses with 1000 bootstrap replicates were carried out using MEGA5 to reconstruct the phylogenetic trees [21].

3. Results

3.1. Comparative analysis of the Os4CL gene family

Rice contains five 4CL isoforms [3]. The amino acid sequence similarities within the Os4CL gene family share a percentage of identity between 56% and 84% (Supplementary Table S5). According to these data, Os4CL3 and Os4CL4 are most similar to each other in sequence, with an identity of 84%. Os4CL2 is the most distantly related to all other isoforms. The particularly high sequence similarity between Os4CL3 and Os4CL4 suggests a recent gene duplication event. Fig. 1A indicates that one copy each segment involved in the duplication event was located on chromosomes II and VI and contains another ORF of unknown function, in addition to Os4CL3/Os4CL4. Fig. 1B illustrates the exon/intron structures of the five Os4CL genes. Os4CL1 and Os4CL5 each contain five exons and four introns, respectively. The positions of the four introns in these genes are conserved, but the introns differ in length and sequence. Os4CL2 contains one additional intron that interrupts the region corresponding to the third exon of Os4CL1 and Os4CL5. The major difference of Os4CL3 and Os4CL4 from Os4CL1 and Os4CL5 is an additional intron in the first exon.

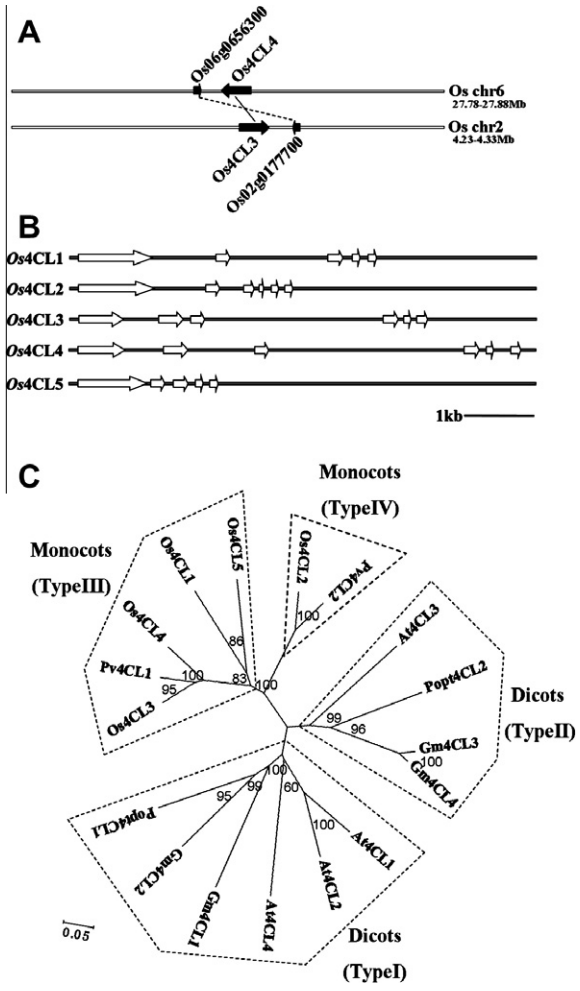

Fig. 1. Gene structure and phylogenetic analysis of the rice 4CL family. (A) Structural features of the five 4CL genes from rice. (B) Relative positions of sequence-related genes on inversely duplicated segments of rice chromosomes II and VI. (C) Phylogenetic analysis of Os4CL proteins with soybean, Arabidopsis, aspen and switchgrass. The branch lengths are proportional to distances, and the values at the interior nodes are the bootstrap percentages derived from 1000 replicates.

Phylogenetic analysis revealed that 4CL genes from dicots and monocots formed separate clusters (Fig. 1C), suggesting that 4CL genes evolved independently in the two groups of plants after the separation between monocots and dicots. In accordance with earlier studies, dicot 4CLs were divided into two groups, namely, type I and type II. Previous studies demonstrated that 4CL genes in type I function in monolignol formation, whereas type II 4CLs activate flavonoid biosynthesis. The 4CLs from monocots can also be classified into two groups, namely, type III and type IV. Os4CL1, Os4CL3, Os4CL4, and Os4CL5 are most closely related to each other within the type III cluster, whereas Os4CL2 is in the divergent type IV cluster.

3.2. Kinetic analysis of recombinant rice 4CL proteins

The enzymes were synthesized in *E. coli* to examine the biochemical properties of the Os4CLs. Four potential substrates, PA, CA, FA, and SA, were used to analyze the enzymatic activity of Os4CLs. The enzyme-kinetic properties of the purified protein (Table 1) confirmed its biochemical function as bona fide 4CLs. The relative Vmax/Km values of recombinant Os4CL1, Os4CL2, Os4CL3, Os4CL4, and Os4CL5 was highest when PA was used as substrate. CA is a relatively poor substrate for recombinant rice 4CLs. It should be noted that SA was not accepted as a substrate under experimental conditions. Previous studies showed that soybean Gm4CL1 and Arabidopsis At4CL4 are the only isoenzymes currently known that can efficiently use SA as a substrate [4,5]. Protein sequence alignments of Gm4CL1 and At4CL4 with other plant isoforms showed that both proteins have a deletion mutation at positions that correspond to Val–338 of *Paulownia tomentosa* 4CL1 [22]. Deleting the corresponding residues from Gm4CL1 and At4CL4 significantly enlarges the substrate-binding pocket, thereby enabling 4CL enzymes to bind to large SA [23]. Sequence comparison of rice 4CLs with Gm4CL1 and At4CL4 revealed one crucial difference: the existence of a single valine residue at the binding site (Fig. 2). The existence of the valine residue might affect rice 4CL activities toward SA.

Kinetic data from single-substrate assays can illustrate the apparent catalytic preferences of 4CLs, but do not consider multi-substrate interactions that can affect activity. Therefore, this study focused on the mixed-substrate enzyme kinetics of rice 4CL proteins. HPLC-UV was used to quantify the produced CoA esters directly from aqueous reaction mixtures. 4CL activity was assayed using three (PA + CA + FA) substrates. The PA:FA:CA product ratio for Os4CL1, Os4CL2, Os4CL3, Os4CL4, and Os4CL5 was 1:0.72:0.06, 1:0:0.09, 1:0.43:0.07, 1:0.45:0.07, and 1:0.48:0.02, respectively (Fig. 3). The data indicated that PA-CoA was the predominant product of rice 4CL. PA and FA strongly inhibited rice 4CL utilization of CA. Thus, CA was a relatively poor 4CL substrate in mixed as single-substrate assays. These results suggested that PA and FA conversion by 4CL are the major metabolic pathways of phenylpropanoid compound biosynthesis in rice.

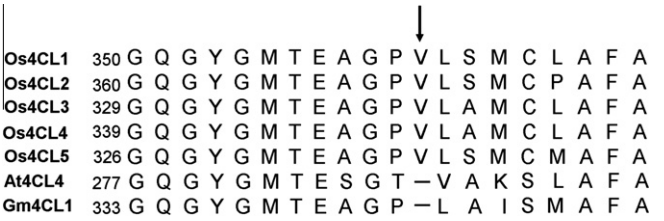

Fig. 2. Partial amino acid alignment of rice (*Oryza sativa*, *Os*) 4CL isoforms 1, 2, 3, 4 and 5, in comparison with *A. thaliana* (*At*) 4CL isoform 4 and soybean (*Glycine max*, *Gm*) 4CL isoform 1. The denoted numbering is according to each single polypeptide, as found in the databases.

Table 1
Kinetic properties of Os4CLs *in vitro*.

Enzyme	Substrate	km (μM)	Vmax (pmol min^{-1} μg^{-1})	Vmax/Km
Os4CL1	4-Coumarate	136 ± 9	1056 ± 66	7.8
	Caffeate	234 ± 12	282 ± 12	1.2
	Ferulate	79 ± 4	552 ± 24	7.0
	Sinapate	No conversion		
Os4CL2	4-Coumarate	103 ± 23	24 ± 6	0.3
	Caffeate	nd		
	Ferulate	180 ± 30	36 ± 12	0.2
	Sinapate	No conversion		
Os4CL3	4-Coumarate	282 ± 32	498 ± 48	1.8
	Caffeate	181 ± 16	210 ± 18	1.2
	Ferulate	380 ± 40	618 ± 60	1.6
	Sinapate	No conversion		
Os4CL4	4-Coumarate	143 ± 12	1284 ± 90	9.0
	Caffeate	154 ± 18	960 ± 102	6.2
	Ferulate	309 ± 19	1698 ± 108	6.4
	Sinapate	No conversion		
Os4CL5	4-Coumarate	212 ± 17	3432 ± 252	16.2
	Caffeate	262 ± 34	2034 ± 234	7.8
	Ferulate	483 ± 41	6282 ± 492	13.0
	Sinapate	No conversion		

Km and Vmax were determined by linear regression of v against v/s (Eadie-Hofstee plot) with at least five data points. The data are mean values from three independent measurements. nd, not determined because of inefficient conversion.

3.3. Developmental and stress-induced expression of rice 4CL genes

The developmental and stress-induced gene expression patterns of five Os4CL genes was examined by qRT-PCR to gain insight into Os4CL potential biological functions. Expression profiles in different rice organs and tissues are shown in Fig. 4A. All five 4CLs were expressed at quantitatively different levels over the course of plant growth, but without any apparent tissue specificity. Os4CL3 was expressed at relatively high levels in most tissues, especially in the hulls where relatively large amounts of lignin accumulated. The Os4CL1 transcript was the least abundant in all organs. A different expression pattern was observed for Os4CL2. The highest relative mRNA amount was detected in the anther, suggesting subfunctionalization in expression patterns. All rice 4CL genes were strongly expressed at the onset of lignin deposition in the plumules and radicles.

Abiotic stresses, such as wounding or irradiation with UV light, also activate phenylpropanoid gene expression [15]. Wound-induced expression data are shown in Fig. 4B. Os4CL3 and Os4CL5 mRNA accumulation was significantly upregulated by wounding, with Os4CL5 levels of 7-fold above the untreated control within 6 h of wounding. Os4CL4 mRNAs also accumulated rapidly and transiently, reaching maximum levels at 1 h after the onset of wounding. Os4CL1 and Os4CL2 expression was downregulated after 1 h in response to wounding, and their expression stayed below the control levels for up to 8 h. These data suggest that Os4CL3, Os4CL4, and Os4CL5 participate in defense against wounding.

Os4CL1, Os4CL3, Os4CL4, and Os4CL5 were downregulated by UV radiation when dark-adapted rice plants were illuminated with UV-containing white light (Fig. 4C). Interestingly, Os4CL2 expression was induced by up to 16-fold at 12 h after the onset of irradiation, and remained high at 24 h after treatment (Fig. 4C). The maximum amount of Os4CL2 mRNA was slightly lower than that of the UV-inducible chalcone synthase gene, OsCHS1, which served as a positive control for UV irradiation efficiency. These results suggested that Os4CL2 was probably involved in UV protective flavonol formation.

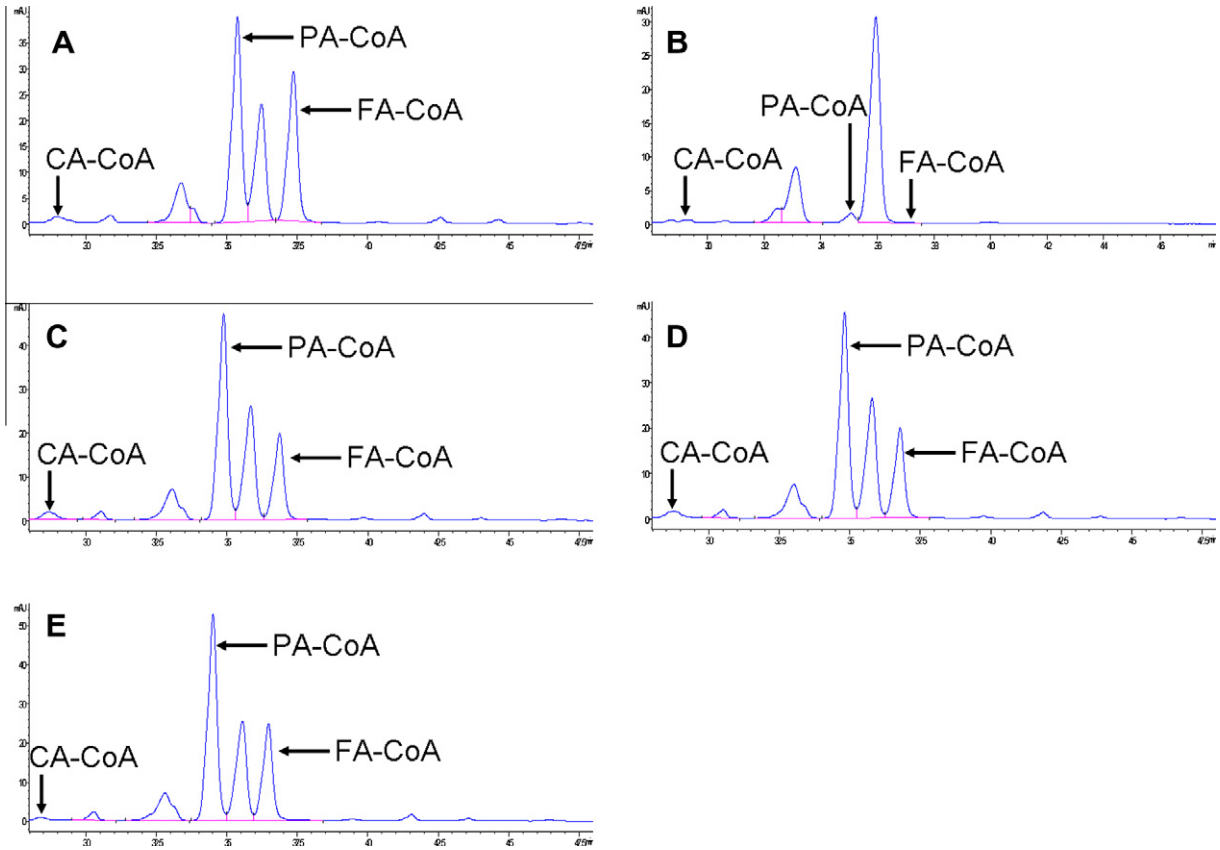

Fig. 3. Representative chromatograms of CoA products from mixed-substrate 4CL assays. CoA ester products from enzymatic reactions using substrate mixtures of equal molar PA, CA and FA were separated by HPLC. Enzyme assays were conducted using recombinant rice 4CL1 (A), 4CL2 (B), 4CL3 (C), 4CL4 (D) and 4CL5 (E).

4. Discussion

4.1. Os4CL2 is the key 4CL isoenzyme involved in flavonoid biosynthesis

Flavonoids are crucial for plant sexual reproduction by promoting pollen tube development [24]. Flavonoids are also essential for protection against damage by UV radiation [25–27]. In this study, we propose that the primary function of Os4CL2 is to channel activated 4-coumarate to chalcone synthase, and subsequently, to different branch pathways of the flavonoid secondary metabolism, leading to flower pigments and UV protective flavonols and anthocyanins. For rice, this proposal is based on the following observations: (a) PA-CoA, the substrate of flavonoid biosynthesis, is the predominant product of Os4CL2 in mixed-substrate assays. (b) Os4CL2 is expressed at relatively high levels in the anthers where flavonoids are essential for pollen germination and tube growth. (c) Os4CL2 is strongly induced by UV light treatments that can stimulate the biosynthesis of UV-absorbing flavonoids. The expression characteristics and enzymatic properties of Os4CL2 are similar to that of Arabidopsis At4CL3 [1], aspen Popt4CL2 [8] and soybean Gm4CL4 [28], which participate in flavonoid biosynthesis, suggesting that Os4CL2 has a similar function.

4.2. 4CL conversion through PA intermediate and 4CL conversion through FA intermediate are two major pathways of lignin biosynthesis in rice

Previous studies demonstrated that Os4CL3 in type III is the key 4CL isoenzyme involved in lignin biosynthesis because the suppression of Os4CL3 expression results in significant lignin reduction and other morphological changes [3]. In addition, the type III 4CL isoform from switchgrass (Pv4CL1) is also described for lignin formation [29]. Os4CL1, Os4CL4, and Os4CL5 belong to the same cluster as Os4CL3 and Pv4CL1, suggesting that they may have a similar function. Consistently, 4-coumarate and ferulate are the preferred substrates for rice 4CL1, 4CL3, 4CL4, and 4CL5 in single- and mixed-substrate assays. Therefore, Os4CL1, Os4CL3, Os4CL4, and Os4CL5 are involved in lignin formation. Based on the mixed-substrate assays, PA-CoA was the predominant product of rice 4CL (Fig. 3). Therefore, 4CL conversion through PA intermediate is a main pathway by which lignin is synthesized in rice. Our findings are consistent with the hypothesis that PA is a main substrate of 4CL for lignin biosynthesis in dicots [30]. Rice 4CL also exhibited a strong preference for FA in mixed-substrate assays (Fig. 3), suggesting that 4CL conversion through FA intermediate is also a pathway for lignin formation. To our knowledge, this study is the first to propose the two major pathways involved in lignin biosynthesis in monocots. These routes were supported by previous studies indicating that the suppression of Os4CL3 expression led to significant increase of PA and FA accumulation in rice [3].

In summary, our data indicated that Os4CL2 is associated with flavonoid biosynthesis, whereas the other 4CL genes (Os4CL1/3/4/5) are involved in channeling hydroxycinnamic acid derivatives for lignin synthesis. However, little is known about the distinct roles of 4CL isoforms in plant defense systems and in lignification in a tissue-specific manner. Therefore, this study provided insights into the different biological functions of Os4CL in rice and in other plants.

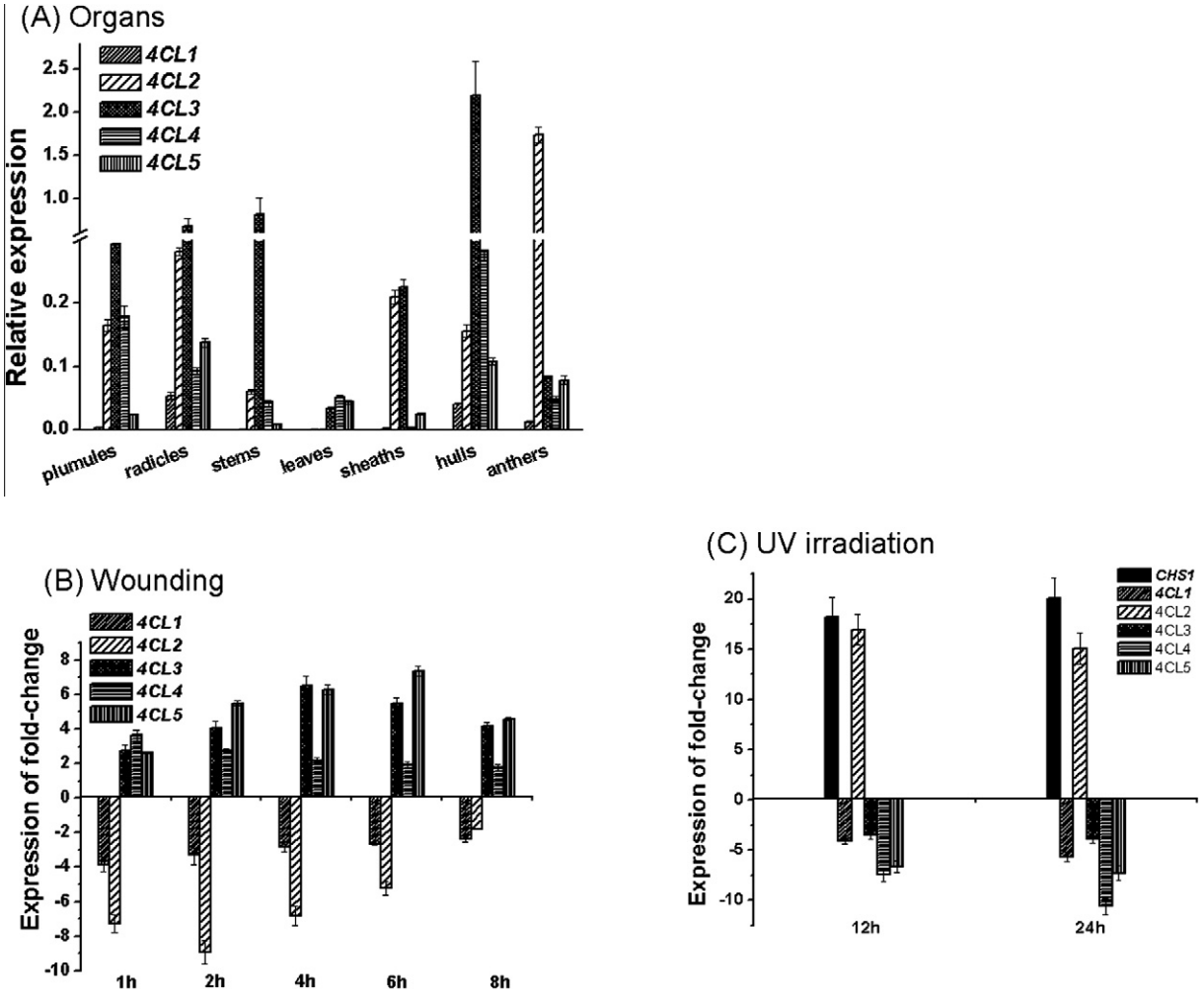

Fig. 4. Organ-, wound-, and UV-light-specific accumulation of *Os4CL* mRNAs. (A) Organ-specific accumulation of *Os4CL* mRNAs. The relative accumulation of Os4CL transcripts was determined by quantitative real-time PCR, and the data were normalized with respect to the *ubq1* gene transcript levels. (B) Quantitative reverse transcriptase polymerase chain reaction analysis of *4CL* gene expression in response to wounding in rice. Data are expressed as fold-change in expression (*y*-axis) relative to unwounded control leaves. Time (in hours) after wounding is given on the *x*-axis. (C) For UV irradiation, dark-adapted rice seedlings were exposed to UV-containing white light for the times indicated and used for RNA extraction and analysis as above. OsCHS1, rice chalcone synthase 1.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.12.019.

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