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Sequence and expression analysis of the C3HC4-type RING finger gene family in rice

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ABSTRACT

C3HC4-type RING finger genes comprise a large family in the plant kingdom and play important roles in various physiologic processes of plant life. In this study, we identified 29 C3HC4-type RING finger family genes in rice (*Oryza sativa*) by database search. Motif analysis revealed the presence of three conserved motifs with unknown functions within the predicted proteins. Promoter analysis found 196 *cis*-elements in the 2-kb upstream regions of these genes, including a stress-responsive element, a hormone-responsive element, and a light-responsive element. In addition, a comprehensive expression analysis of these genes has been performed using microarray data obtained from 27 tissues or organs of three rice genotypes, Minghui 63, Zhenshan 97, and Shanyou 63. Real-time PCR analysis confirmed that five C3HC4-type RING finger genes are preferentially expressed in reproductive tissues or organs such as stamen, panicle, and endosperm. Expression analysis of C3HC4-type RING finger genes under abiotic stresses suggests that twelve genes are differentially regulated by hormones or stress in rice seedlings. These results would be useful for elucidating their roles in the growth, development, and stress response of the rice plant.

1. Introduction

RING finger is a member of the Zinc finger domains, which were first identified as a DNA-binding motif in the transcription factor TFIIIA from *Xenopus laevis*. In addition to DNA, they also bind RNA, protein, or lipid substrates (Berg and Shi, 1996). The RING domain was originally named after the acronym for the first RING finger gene (Really Interesting New Gene). It is a relatively small protein motif and consists of four pairs of ligands binding two ions (Joazeiro and Weissman, 2000).

RING finger proteins are involved in numerous cellular processes including transcription, signal transduction, and recombination. Functions attributed to the RING domain itself include protein–protein interaction and ubiquitination (Borden and Freemont, 1996; Lorick et al., 1999). Most RING finger proteins are E3 ubiquitin ligases (Stone et al., 2005) that mediate the transfer of the ubiquitin to target proteins and play important roles in diverse aspects of cellular regulations in plants (Ciechanover, 1998; Hershko and Ciechanover, 1998; Callis and Vierstra, 2000; Ben-Neriah, 2002).

Based on the type of cysteine and histidine residue combination, the RING finger domain can be classified into canonical and modified RING fingers. The C3HC4-type RING finger subclass, which is also designated as RING-HC, belongs to the canonical RING finger. Another member of the canonical RING finger is the C3H2C3 type, which is also called the RING-H2 subclass. The consensus sequence of C3HC4-type RING finger can be described as Cys-X2-Cys-X(9–39)-Cys-X(1–3)-His-X(2–3)-Cys-X2-Cys-X(4–48)-Cys-X2-Cys, where phenylalanine and proline residues are highly conserved but not invariable, and the loops vary in length. Whereas other characterized RING domains contain a His at metal ligand position 4, the C3HC4 type differs with the presence of a Cys residue at metal ligand position 5 (Freemont, 1993; Lovering et al., 1993). Different subclasses of the RING finger domain determine specificity toward different E2 ubiquitin-conjugating enzymes (Huibregtse et al., 1995).

C3HC4-type RING finger proteins have been studied on a genomic scale in *Arabidopsis* (Stone et al., 2005). *Arabidopsis* RING-HC proteins with predicted or known biologic function include *AtCOP1* (light) and *AtCOP1*-interacting protein 8 (*AtCIP8*; photomorphogenesis) (von Arnim and Deng, 1993; Hardtke et al., 2002), *AtTED3* (light signaling) (Pepper and Chory, 1997), *AtRMA1* (secretory pathway) (Matsuda et al., 2001), *AtPEX10* and *AtPEX12* (peroxisome biogenesis) (Schumann et al., 2003; Fan et al., 2005), *AtPRT1* (N-end rule pathway) (Potuschak et al., 1998; Stary et al., 2003), *AtXB3* (root development) (Wang et al., 2006), *AtHUB1* and *AtHUB2* (chromatin modifications) (Liu et al., 2007b), and *AtSDIR1* (stress tolerance) (Zhang et al., 2007).

Until now only a few C3HC4-type RING finger genes have been identified in rice, including *OsCOP1* (*OsRHC11*), *OsCOIN1* (*OsRHC13*), *OsXB3.1* (*OsRHC24*), and *OsRHC1*. OsCOP1 is a component of the signal

Abbreviations: aa, amino acid; ABA, abscisic acid; ABRE, ABA-responsive element; BR, Brassinosteroid; DRE, dehydration-responsive element; GA_3, gibberellin; H_2O_2, hydrogen peroxide; IAA, indoleacetic acid; JA, Jasmonic acid; KOME, Knowledge-Based *Oryza* Molecular Biological Encyclopedia; KT, cytokinin; LTRE, low-temperature-responsive element; MEME, Multiple Em (Expectation Maximization) for Motif Elicitation; NaCl, sodium chloride; *OsRHC*, rice C3HC4-type RING finger; PCR, polymerase chain reaction; TIGR, the Institute of Genomic Research; UPS, ubiquitin proteasome system; UTR, untranslated region.

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transduction chain linking light signals to plant development. This best studied C3HC4-type RING finger protein (von Arnim and Deng, 1993; Raghuvanshi et al., 2001; Tsuge et al., 2001) functions as an E3 ubiquitin ligase, which targets photomorphogenesis-promoting transcription factors for ubiquitylation and degradation. In the dark, OsCOP1 accumulates in the nucleus where it is required for the degradation of the OsHY5 protein, a positive regulator of photomorphogenesis. In the light, OsCOP1 is excluded from the nucleus and the constitutively nuclear OsHY5 protein can accumulate. It has been demonstrated that overexpression of *OsCOIN1* increased tolerance to chilling, salt, and drought in rice, and the transgenic lines also displayed up-regulation of *OsP5CS* (a known cold-induced gene) expression and an increased cellular proline level (Liu et al., 2007a). Two other C3HC4-type RING finger genes, *OsRHC24* and *OsRHC1*, are related to disease resistance (Wang et al., 2006; Cheung et al., 2007).

Rice is one of the major crops in the world and it is a model species for the functional genomic study of monocotyledon plants. To date, the function of C3HC4-type RING finger genes in rice is much less understood compared to that in dicotyledonous model plants such as *Arabidopsis* and tobacco. In this study, 29 C3HC4-type RING finger genes were identified in the rice genome and a global overview of this gene family was given including the phylogenetic relationship, motif compositions, and possible *cis*-elements. The expression profiles of this gene family in the entire rice life cycle and under different hormone treatment conditions were also analyzed using data from microarray and real-time PCR. Such a comprehensive analysis of these C3HC4-type RING finger genes may provide important clues for understanding their diverse roles in the growth and development of the rice plant.

2. Materials and methods

2.1. Database search

The protein family ID PF00097 was queried in the database (Release 5) of the Institute of Genomic Research (TIGR) (http://www.tigr.org) for obtaining sequences of the C3HC4-type RING finger family genes in rice. All the corresponding protein sequences of the putative C3HC4-type RING finger family members were downloaded and confirmed with the Pfam database (http://www.sanger.ac.uk/Software/Pfam/search.shtml). Information about the chromosomal localization, amino acid (aa) length and full-length cDNA accessions for each gene was obtained from TIGR and the Knowledge-Based *Oryza* Molecular Biological Encyclopedia (KOME) (http://cdna01.dna.affrc.go.jp/cDNA).

2.2. Motif confirmation and subcellular location

Alignment of the protein sequences was performed using CLUSTAL_X version 1.83 (Thompson et al., 1997). A phylogenetic tree was constructed using MEGA4 (Tamura et al., 2007). Bootstrap testing was performed with 1000 resamplings. The potential motifs in the putative C3HC4-type RING finger family gene sequences were predicted by the Multiple Em (Expectation Maximization) for Motif Elicitation (MEME) program version 3.5.4 (Bailey and Elkan, 1994). The cellular localization of each C3HC4-type RING finger protein was identified by PSORT analysis (http://psort.nibb.ac.jp/).

2.3. Promoter sequence analysis

Promoter sequences (2 kb upstream of the translation start codon) for all C3HC4-type RING finger family members were obtained from TIGR (http://www.tigr.org/tdb) and subjected to scan for plant *cis*-acting regulatory DNA elements (PLACE) (http://www.dna.affrc.go.jp/PLACE/signalscan.html) to identify all the plant *cis*-elements with more than 6 bp (Higo et al., 1999).

2.4. Expression profile analysis

Expression profile data were obtained by Affymetrix Rice GeneChip microarray from CREP (http://crep.ncpgr.cn), a database of the rice transcriptome project. In this study we singled out the expression signal values of the rice C3HC4-type RING finger genes from the database for 27 tissues or organs and for hormonal treatments (naphthlcetic acid [NAA], gibberellin [GA_3], or cytokinin [KT]) in three rice genotypes (Minghui 63, Shanyou 63, and Zhenshan 97). A gene was regarded as expressed in a tissue if its average expression signal value from the CREP database was greater than 50. To identify the tissue-preferential expressed genes, we selected one tissue and then compared it with all other 26 tissues by performing Student's *t* test in each genotype separately. A gene expression value in a tissue in three genotypes with *P* value less than 0.05 and expression values more than twofold higher than in all other tissues were considered to be expressed preferentially.

2.5. Plant growth and stress treatment

To identify tissue-preferentially expressed genes, seedlings of Minghui 63 were grown under normal conditions. Eleven tissues or organs in the entire rice life cycle were collected for real-time PCR analysis. To verify the expression profiles of C3HC4-type RING finger genes under hormone or different abiotic stress conditions, the seedlings of Zhonghua 11 at the four-leaf stage were cultured under stress conditions (salt, drought, and cold) and chemical treatments (abscisic acid [ABA], NAA, GA_3, KT and hydrogen peroxide [H_2O_2]). For high salinity treatment, NaCl was added at a final concentration of about 200 mM. For cold stress, the seedlings were transferred to a growth chamber at 4 °C with 12 h light/12 h dark for 5 days and then

Table 1
The primers used for real-time PCR of C3HC4-type RING finger genes in rice.

Gene	Primers for real-time PCR (5′–3′)
OsRHC2	Forward: TTCGTTGGATGTTCGCAGAA
	Reverse: TATCGACATCCGCAACTTTGG
OsRHC3	Forward: GACCGCCGGAATCCTATTTC
	Reverse: GTTGCCAATGCTCTCACCAT
OsRHC7	Forward: ACAAGCCTGACCACGACTTCTC
	Reverse: CACAAATAGCAGCAGCGGC
OsRHC11	Forward: ATAATCCTGGGTCGAGCCAC
	Reverse: TATGGTGATCAGCAGAACCCAC
OsRHC12	Forward: CTACTCCCTCAATCGCCAGC
	Reverse: CCTCTGGCAGCTCAAGGAAC
OsRHC13	Forward: CCAAAGTTGCGGATGTCGAT
	Reverse: CAACTGCCATGCTTTCGTTC
OsRHC14	Forward CGTGTGGATCTATGCGACAA
	Reverse TGGGTGAAGAAGACGACGAA
OsRHC15	Forward: TGGAA GTGGAGGAAGGGATCT
	Reverse: TGCTCCTTGCTTGTTCGCTT
OsRHC16	Forward: GCCGTGGTTGTAATCAAGGA
	Reverse: TCCTTTGCTCTTCTTAGGCTGC
OsRHC18	Forward: ACCAGCGGCAGATTTCAGAC
	Reverse: CCTGCTGGTTGATGTTCACC
OsRHC19	Forward: CAGATCATCAGATGGAGGGC
	Reverse: TTCCTGTGCTGCTGACATGG
OsRHC20	Forward: CGCCGCATTTCGTAGGAATT
	Reverse: CCAGCGATTTCTCCGATGAT
OsRHC22	Forward: TGCAGATTCCGGGAGAAG
	Reverse: GCCGATGATGATGTGCTG
OsRHC24	Forward: GCCTCGTCAATTGCTTCAGG
	Reverse: CCCTATACGAAGACGACCGC
OsRHC25	Forward: TCTAGCTGCAACTGAGGTGG
	Reverse: CCCCTTCTTTGTCCTGACTG
OsRHC27	Forward: TGCCTTGCTCTCATGACTTCC
	Reverse: CCCTAAGCCATTCAGAGATGCA
OsRHC28	Forward: GACAAAGACAGGAAGCGGC
	Reverse: GGATGAATGCCACCTCTGCT

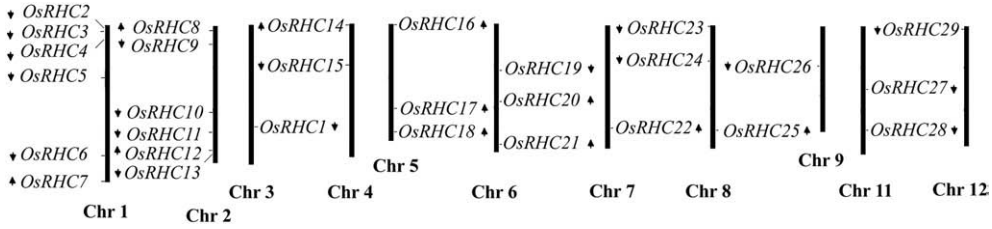

Fig. 1. Genomic distribution of C3HC4-type RING finger genes on rice chromosomes. The arrows next to gene names show the direction of transcription.

back to normal growth conditions for recovery. Drought stress was induced by stopping watering at about 2 weeks before flowering and leaves were sampled according to the degree of leaf-rolling. Chemical treatments including NAA (200 μL/L), GA_3 (200 μL/L), KT (200 μL/L), ABA (200 μL/L), and H_2O_2 (500 μL/L) were also applied to the seedlings at the four-leaf stage.

2.6. Real-time PCR analysis

For real-time PCR analysis, first-strand cDNA was synthesized from DNaseI-treated total RNA using Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Real-time PCR was performed in an optical 96-well plate with an ABI PRISM 7500 Real-time PCR System (Applied Biosystems, Carlsbad, CA). Each reaction contains 10 μL 2× SYBR Green Master Mix Reagent (Applied Biosystems), 1.0 μL cDNA sample, and 200 nM of gene-specific primer in a final volume of 20 μL. The thermal cycle used was as follows: 95 °C for 3 s; 45 cycles of 95 °C for 5 s, 60 °C for 34 s. Rice *Actin1* gene (accession number X16280) was used as internal control (The primers were 5′-TGGCATCTCTCAGCACATTCC-3′ and 5′-TGCACAATGGATGGGTCAGA-3′). The gene-specific primers are listed in Table 1. The relative expression levels were determined as described previously (Livak and Schmittgen, 2001).

3. Results

3.1. The C3HC4-type RING finger family members in rice

To identify the C3HC4-type RING finger genes in rice, the protein family ID PF00097 was used to search the rice genome annotation database in TIGR. The domain score greater than trusted cut-off and the total HMM score greater than 50 were used to specify the search criteria by the TIGR database. Twenty-three distinct loci with 53 gene models encoding putative C3HC4-type RING finger proteins were identified in the rice genome. BLAST analysis against the Pfam database showed that 22 of them belong to the C3HC4-type RING

Table 2
General information about C3HC4-type RING finger protein-encoding genes in rice.

Group	Gene name[a]	Probeset ID[b]	TIGR locus[c]	BAC/PAC[d]	Fl-cDNA accession no.[e]	aa[f]	P.L.[g]	Gene reported[h]
Group I	*OsRHC7* (*OsXB3.3*)	Os.32105.1.S1_at	Os01g74320	AP003627	AK106014	446	Cytoplasm	
	OsRHC12 (*OsXB3.2*)	Os.47766.1.S1_at	Os02g54860	AP003984	AK120632	531	MMS	
	OsRHC20	OsAffx.15738.1.S1_at	Os06g34400	AP008212	NF	424	PM	
	OsRHC23	Os.50424.1.S2_at	Os08g01040	AP005406	AK121153	610	PM	
	OsRHC24 (*OsXB3.1*)	Os.5876.2.S1_at	Os08g15840	AP005151	AK120159	495	Nucleus	Wang et al. (2006)
	OsRHC27	OsAffx.19490.1.S1_at	Os11g04680	AC151480	NF	270	Cytoplasm	
Group II	*OsRHC2* (*OsCOIN2*)	Os.32481.1.S1_s_at	Os01g01420	AP002818	AK072797	363	Nucleus	
	OsRHC11 (*OsCOP1*)	Os.3433.1.S1_at	Os02g53140	AP004058	AK112098	604	Nucleus	Tsuge et al. (2001)
	OsRHC13 (*OsCOIN1*)	Os.10314.1.S1_a_at	Os02g55200	AP004094	AK071071	365	CS	Liu et al. (2007a)
	OsRHC29	Os.14655.1.S1_x_at	Os12g04650	AL928783	AK101391	171	Nucleus	
Group III	OsRHC1	Os.10336.1.S1_at	Os03g49900	AC087797	AK065293	473	PM	Cheung et al. (2007)
	OsRHC3	Os.30607.1.S1_at	Os01g03100	AP002487	AK108601	269	PM	
	OsRHC4	Os.1438.1.S1_at	Os01g06590	AP002539	AK073728	501	Nucleus	
	OsRHC5	Os.33687.1.S1_at	Os01g19800	AP003206	AK099496	220	Cytoplasm	
	OsRHC6	Os.25849.3.S1_x_at	Os01g66970	AP003407	AK073332	219	CTM	
	OsRHC8	Os.27262.1.S1_a_at	Os02g03950	AP004150	AK070078	272	Cytoplasm	
	OsRHC9	Os.38687.1.S1_at	Os02g06920	AP004863	AK101610	487	PM	
	OsRHC10	Os.18677.1.S1_at	Os02g36740	AP006070	AK071239	250	Cytoplasm	
	OsRHC14	Os.7825.1.S1_at	Os04g01490	AL662992	AK071063	459	PM	
	OsRHC18	Os.55141.1.S1_at	Os05g47900	AC135925	AK107568	252	Nucleus	
	OsRHC19	Os.49736.1.S1_at	Os06g23274	AP003952	AK121342	497	ER	
	OsRHC21	Os.17420.1.S1_at	Os06g46366	AP004989	AK100432	483	PM	
	OsRHC22	Os.41109.1.S1_x_at	Os07g43740	AP004339	AK120260	185	CTM	
	OsRHC25	Os.5671.1.S1_at	Os08g33860	AP005159	AK071857	276	Nucleus	
	OsRHC26	Os.51930.1.S1_at	Os09g12720	AP004011	AK064311	532	Nucleus	
	OsRHC28	Os.6005.1.S1_at	Os11g38800	AC109365	AK071098	278	Nucleus	
Group IV	*OsRHC15*	OsAffx.3855.1.S1_at	Os04g22240	AP008210	AK242034	376	Nucleus	
	OsRHC16	Os.49723.1.S1_at	Os05g01230	AC084818	AK101136	789	Nucleus	
	OsRHC17	Os.54186.1.S1_at	Os05g37900	AC097176	AK102413	244	Nucleus	

NF, not found; PM, plasma membrane; CTM, chloroplast thylakoid membrane; ER, endoplasmic reticulum; MMS, mitochondrial matrix space; CS, chloroplast stroma.
[a] Systematic designation given to rice C3HC4-type RING finger genes in this study.
[b] Probeset ID of C3HC4-type RING finger genes obtained from CREP (http://crep.ncpgr.cn).
[c] Locus ID of C3HC4-type RING finger genes.
[d] BAC/PAC of the C3HC4-type RING finger protein-encoding genes.
[e] Full-length cDNA accession number of C3HC4-type RING finger genes obtained from KOME.
[f] Protein length (number of amino acids) obtained from TIGR.
[g] Localization of C3HC4-type RING finger protein supported by PSORT (http://psort.nibb.ac.jp).
[h] *OsRHC* genes reported in earlier studies.

finger family. Seven previously reported C3HC4-type RING finger genes were also analyzed in this study though they were not included in the 22 putative C3HC4-type RING finger genes we identified because their HMM score was less than 50. The BAC or PAC clones carrying the 29 C3HC4-type RING finger protein-encoding genes were identified based on the information of rice chromosomal pseudomolecules available at TIGR. The chromosomal locations and directions of transcription of C3HC4-type RING finger genes are shown in Fig. 1. The 29 C3HC4-type RING finger genes are distributed on all of the rice chromosomes except chromosome 10: 6 genes on chromosome 1, 6 genes on chromosome 2, 3 genes each on chromosome 5, 6, and 8, 2 genes each on chromosomes 4 and 11, and a single gene each on chromosomes 3, 7, 9, and 12 (Fig. 1). All of the 29 genes were given systematic names from *OsRHC1* to *OsRHC29* in this study (Table 2).

Search of the KOME database obtained significant matches with 27 full-length cDNA. All the C3HC4-type RING finger proteins had a typical C3HC4-type RING finger domain (PF00097). Twelve of the 29 C3HC4-type RING finger proteins (OsRHC2, OsRHC4, OsRHC11, OsRHC15, OsRHC16, OsRHC17, OsRHC18, OsRHC24, OsRHC25, OsRHC26, OsRHC28 and OsRHC29) showed localization in the nucleus by a PSORT analysis (http://psort.nibb.ac.jp/), whereas 7 proteins were located on plasma membranes (OsRHC1, OsRHC3, OsRHC9, OsRHC14, OsRHC20, OsRHC21 and OsRHC23). Five proteins (OsRHC5, OsRHC7, OsRHC8, OsRHC10 and OsRHC27) showed localization in cytoplasm. The remaining 5 proteins showed localization on three different organelles: two on chloroplast thylakoid membrane (OsRHC6 and OsRHC22), one on endoplasmic reticulum (OsRHC19), one on the mitochondrial matrix space (OsRHC12), and one on chloroplast stroma (OsRHC13) (Table 2).

The full-length cDNA sequences were compared with the corresponding genomic DNA sequences to determine the numbers and positions of exons and introns within each C3HC4-type RING finger gene by using GSDS (http://gsds.cbi.pku.edu.cn/chinese.php) (Guo et al., 2007). The coding sequences of all the C3HC4-type RING finger genes are disrupted by introns, with numbers varying from 0 to 13 (Fig. 2). Based on the number of introns, the C3HC4-type RING finger genes can be classified into two groups: an intron-poor group (for 7 genes, *OsRHC7*, *OsRHC17*, *OsRHC20*, *OsRHC22*, *OsRHC26* *OsRHC27* and *OsRHC29*, each with 0–2 introns) and an intron-rich group (for all the other genes with the number of introns varying from 5 to 13).

3.2. Phylogenetic analysis

The full-length protein sequences of the C3HC4-type RING finger family members in rice (29 genes) and in *Arabidopsis* (21 genes) were used to construct the joint unrooted phylogenetic tree (Fig. 3). The 21 published *Arabidopsis* proteins, such as AtCOP1, AtHUB family and AtPEX family, were included as reference sequences. Our results suggested that the C3HC4-type RING finger family proteins may be classified into four major groups (I, II, III and IV) with well-supported bootstrap values. Group I contained 11 members (6 members of rice and 5 members of *Arabidopsis*), group II contained 8 members (4 members of rice and *Arabidopsis*, respectively), group III contained 20 members (16 members of rice and 4 members of *Arabidopsis*), and group IV had 11 members (3 members of rice and 8 members of *Arabidopsis*). Eight rice and *Arabidopsis* C3HC4 RING finger gene pairs with very close phylogenetic relationships were found in the phylogenetic tree, *AtXBT32* and three *OsXB3*

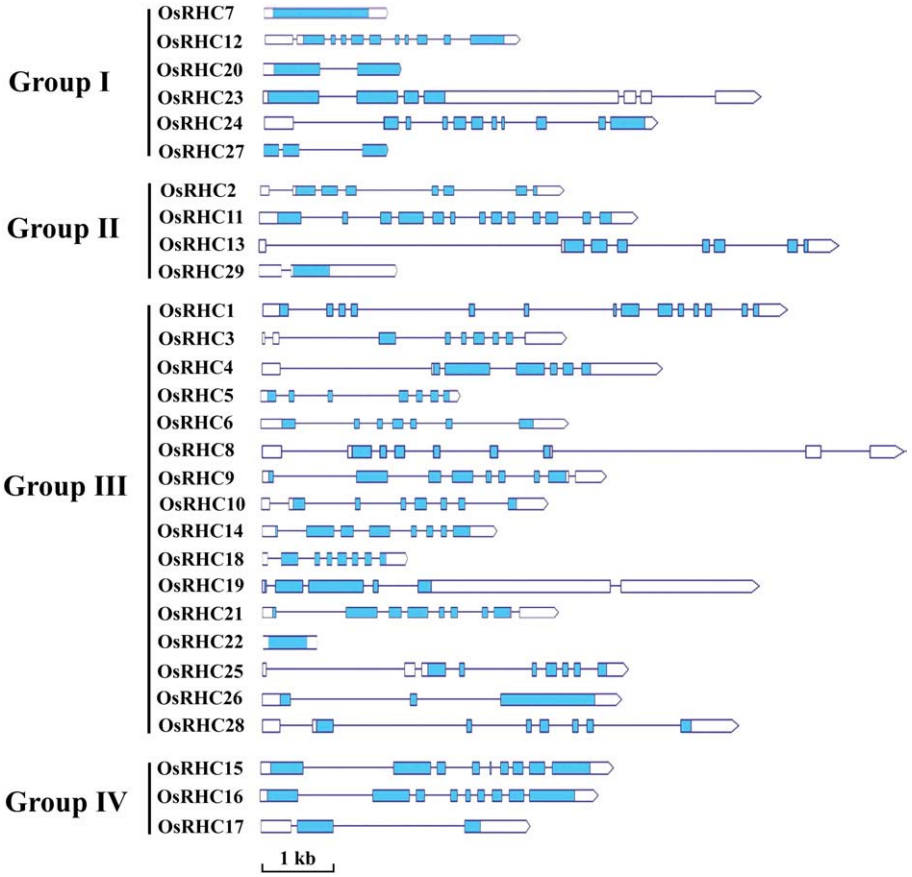

Fig. 2. The GSDS (http://gsds. cbi.pku.edu.cn/chinese.php) output of the C3HC4-type RING finger genes structure in rice. The untranslated region (UTR), exons, and introns are indicated by white rectangles, blue rectangles, and lines, respectively.

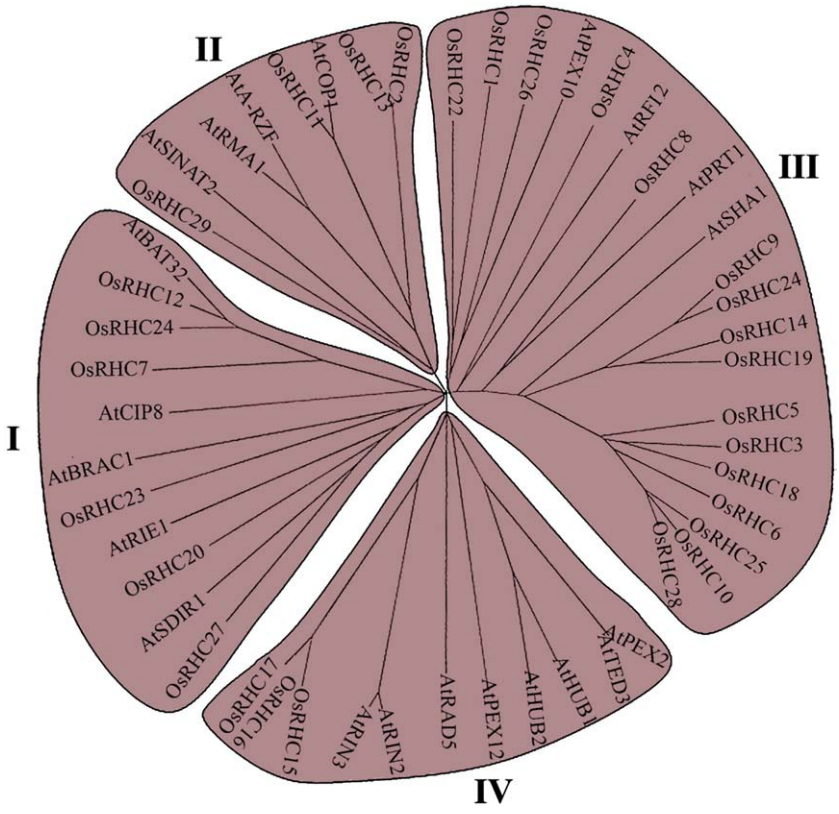

Fig. 3. Phylogenetic relationship between C3HC4-type RING finger genes in rice. The joint unrooted tree was generated using MEGA4 by the neighbor-joining method. Bootstrap values from 1000 replicates were indicated at each branch.

genes (*OsRHC7*, *OsRHC12*, and *OsRHC24*), *AtBRCA1* and *OsRHC23*, *AtSDIR1* and *OsRHC27*, *AtCOP1* and *OsRHC11*, *AtSINAT2* and *OsRHC29*, *AtPEX10* and *OsRHC26*, *AtRFI2* and *OsRHC4*, *AtPRT1* and *OsRHC8* (Fig. 3).

Furthermore, we examined the protein subcellular localization by PSORT analysis of the 8 rice and *Arabidopsis* C3HC4 RING finger gene pairs with very close phylogenetic relationships. Among the 8 gene pairs, AtCOP1 and OsRHC11 in group II were both located in

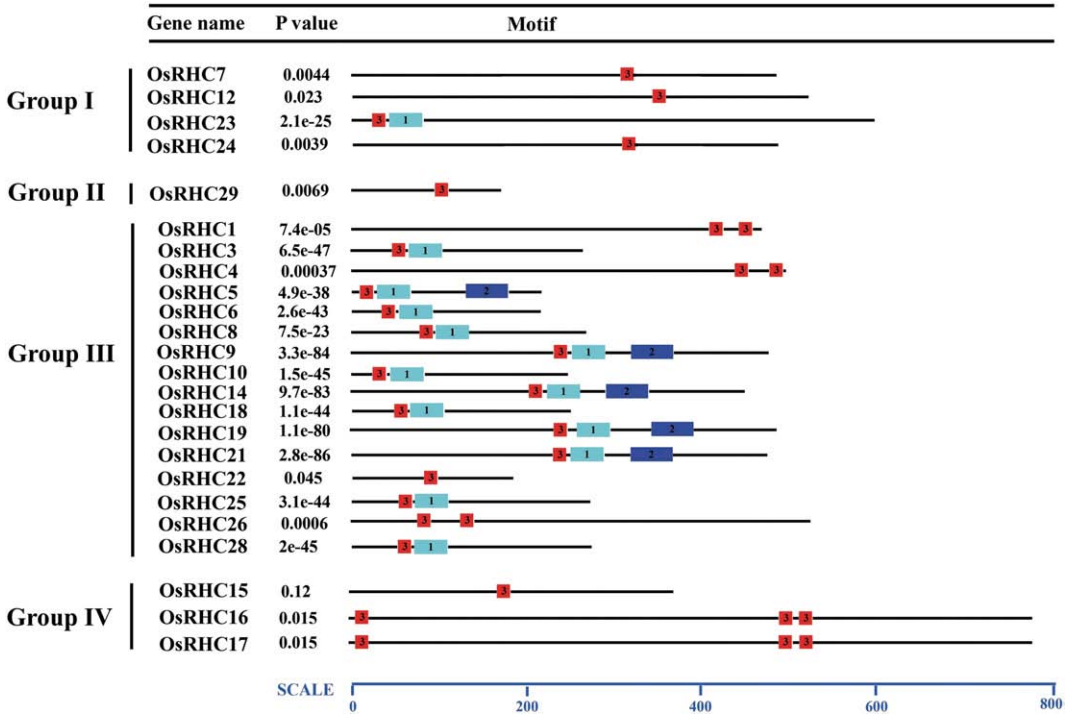

Fig. 4. Organization of putative motifs in C3HC4-type RING finger genes identified by MEME. Numbered color boxes represent different putative motifs. Motifs 1, 2, and 3 are indicated by the light green, blue, and red box, respectively. Names of all the members and combined *P* values are shown on the left side of the figure and motif sizes are indicated at the bottom of the figure.

the nucleus, and 2 rice RING finger proteins, which belonged to group III, had the same localization in the nucleus with their *Arabidopsis* homologues (AtPEX10 and OsRHC26, AtRFI2 and OsRHC4). However, the other 5 rice proteins showed different localizations with their *Arabidopsis* counterparts. For example, AtPRT1 and OsRHC8 were localized in chloroplast and cytoplasm, respectively,

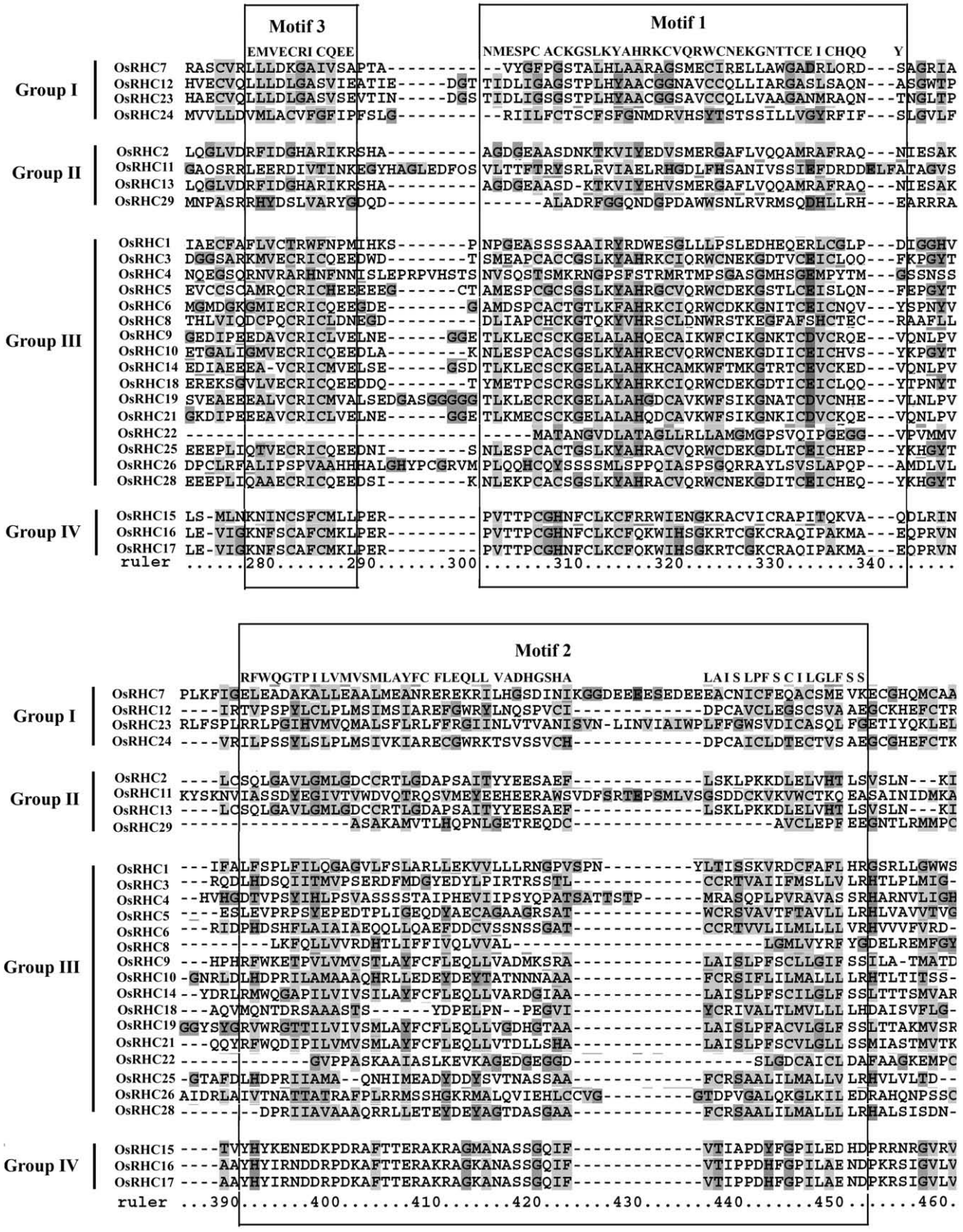

Fig. 5. Protein sequence alignment of three putative conserved motifs identified in C3HC4-type RING finger genes in rice obtained by MEME. Each of the three motifs had 39, 49, and 11 conserved aa residues, respectively.

though they were also in group III. Our results indicated that the same phylogenetic grouping by sequence similarity did not mean the same subcellular localizations.

3.3. Motif analysis

Three putative conserved motifs were identified in the C3HC4-type RING finger family members based on the protein sequence alignment using the MEME software (Fig. 4). Five genes were not mentioned here because two did not have reported full-length cDNA in the KOME database and three could not be confirmed by motif search. Among these three motifs, motif 1 has 39 conserved aa residues, motif 2 has 49 conserved aa residues, and motif 3 has 11 conserved aa residues. Motif 3 is the most conserved and is present in all the C3HC4-type RING finger family members (Fig. 5). Two genes without full-length cDNA in the KOME database were not mentioned here. Five genes (*OsRHC5*, *OsRHC9*, *OsRHC14*, *OsRHC19* and *OsRHC21*) contained all the three motifs. In addition, 8 genes (*OsRHC3*, *OsRHC6*, *OsRHC8*, *OsRHC10*, *OsRHC18*, *OsRHC23*, *OsRHC25* and *OsRHC28*) contained motif 1 and motif 3, and the remaining 11 genes contained only motif 3. According to the numbers in motif 3, the 11 genes could be divided into 3 subgroups: motif 3 appeared once in 6 genes (*OsRHC7*, *OsRHC12*, *OsRHC15*, *OsRHC22*, *OsRHC24* and *OsRHC29*), twice in 3 genes (*OsRCH1*, *OsRHC4* and *OsRHC26*), and thrice in 2 genes (*OsRHC16* and *OsRHC17*) (Fig. 4).

3.4. In silico cis-element analysis of C3HC4-type RING finger genes

To identify putative *cis*-acting regulatory DNA elements existing in C3HC4-type RING finger genes, the 2-kb upstream regions of the 29 C3HC4-type RING finger genes were subjected to the PLACE database search, and 196 putative *cis*-elements with more than 6 bp in length were identified (Supplementary Table 1). Among these 196 putative *cis*-elements, 59 were found with three or more copies in at least 3 of the 29 C3HC4-type RING finger family members and the 10 most enriched *cis*-elements were: CGCGBOXAT, EBOXBNNAPA, EECCRCAH1, GT1CONSENSUS, GT1GMSCAM4, MYBCORE, MYCCONSENSUSAT, POLASIG1, SEF4MOTIFGM7S and TATABOX5. In fact there are some well-characterized stress-responsive *cis*-elements among the 59 *cis*-elements. For example, dehydration-responsive element (DRE) and low-temperature-responsive element (LTRE) (Shinwari et al., 1998; Shinozaki and Yamaguchi-Shinozaki, 2000; Narusaka et al., 2003) have been found in the promoter region of 19 genes. In addition, the 2 kb upstream of 29 genes contained 4 hormone-responsive elements (ABRE, ARFAT, WBBOXPCWRKY1 and PYRIMIDINEBOXOSRAMY1A). Light-responsive *cis*-element BOXIINTPATPB was found in promoter region of 20 genes.

3.5. Expression profiles of C3HC4-type RING finger genes in different tissues or organs of rice

So far little is known about the specificity of tissues or organs of the plant C3HC4-type RING finger genes, which may elucidate their functions in detail, because the multigene family members have not been systemically examined. Thus, we checked our whole genome expression profile data of rice for 27 tissues in three genotypes (Minghui 63, Zhenshan 97, and Shanyou 63) (Wang et al., unpublished data). We found that 5 genes (*OsRHC15*, *OsRHC16*, *OsRHC18*, *OsRHC20* and *OsRHC25*) were preferentially expressed in some tissues or organs and thus exhibited tissue- or organ-preferential expression patterns and 23 genes were widely expressed in almost all tissues. The other one gene (*OsRHC3*) had the expression level less than 50 in most tissues or organs. It is worth mentioning that the average expression signal value of *OsRHC1* and *OsRHC2* were high in the corresponding tissues of all three genotypes (Fig. 6).

Real-time PCR was then performed to confirm the tissue-preferential expression patterns of the 5 genes in 11 representative tissues from an entire life cycle of rice (Fig. 7). Generally, the real-time PCR results matched very well with the DNA microarray data. The results showed that the expression of *OsRHC15* was not detected in almost all tissues or organs except in spikelet of 3 days after pollination and in endosperm of 7 days after pollination. Similarly, *OsRHC16* was highly expressed in endosperm of 7 days after pollination. *OsRHC18* had the highest expression level in stem of the

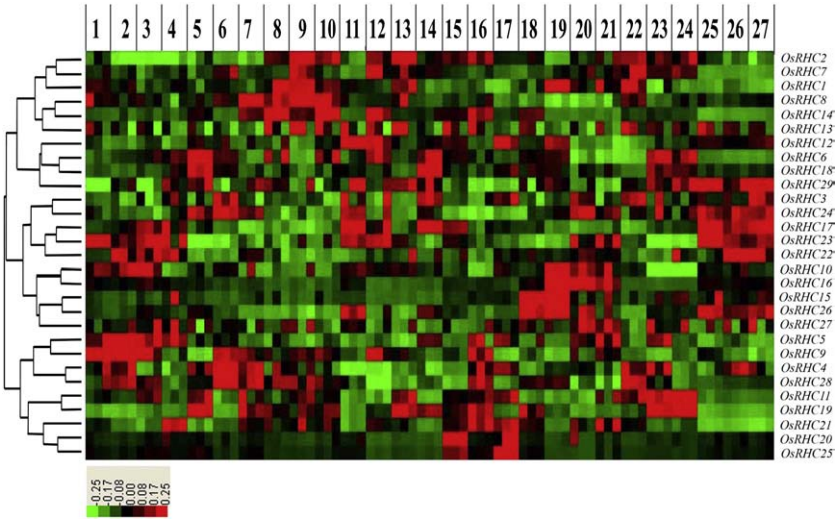

Fig. 6. Hierarchical cluster display of C3HC4-type RING finger genes in rice based on the average signal values in 27 tissues. Numbers from 1 to 27 above the cluster represent the 27 rice tissues representing the entire life cycle. Each tissue has three parallel displays representing Minghui 63 (MH), Shanyou 63 (SY) and Zhenshan 97 (ZS), respectively. The color scale representing average signal values is shown at the bottom. The tissue names are as follows: 1 = callus (at 15 days after subculture stage); 2 = resistance callus (at screening stage); 3 = callus (at 5 days after regeneration stage); 4 = seed (at 72 h of imbibitions stage); 5 = shoot (seedling with 2 tillers); 6 = root (seedling at trefoil stage); 7 = leaf (young panicle at secondary branch primordium differentiation stage III); 8 = sheath (young panicle at secondary branch primordium differentiation stage III); 9 = leaf (4–5 cm young panicle); 10 = sheath (4–5 cm young panicle); 11 = panicle (4–5 cm young panicle); 12 = stem (5 days before heading); 13 = flag leaf (5 days before heading); 14 = stem (at heading stage); 15 = panicle (at heading stage); 16 = hull (one day before flowering); 17 = stamen (one day before flowering); 18 = spikelet (3 days after pollination); 19 = endosperm (7 days after pollination); 20 = endosperm (14 days after pollination); 21 = endosperm (21 days after pollination); 22 = flag leaf (14 days after heading); 23 = seedling (3 days after sowing); 24 = seedling (trefoil stage); 25 = panicle (secondary branch primordium differentiation stage III); 26 = panicle (pistil/stamen primordium differentiation stage IV); 27 = panicle (pollen-mother cell formation stage V).

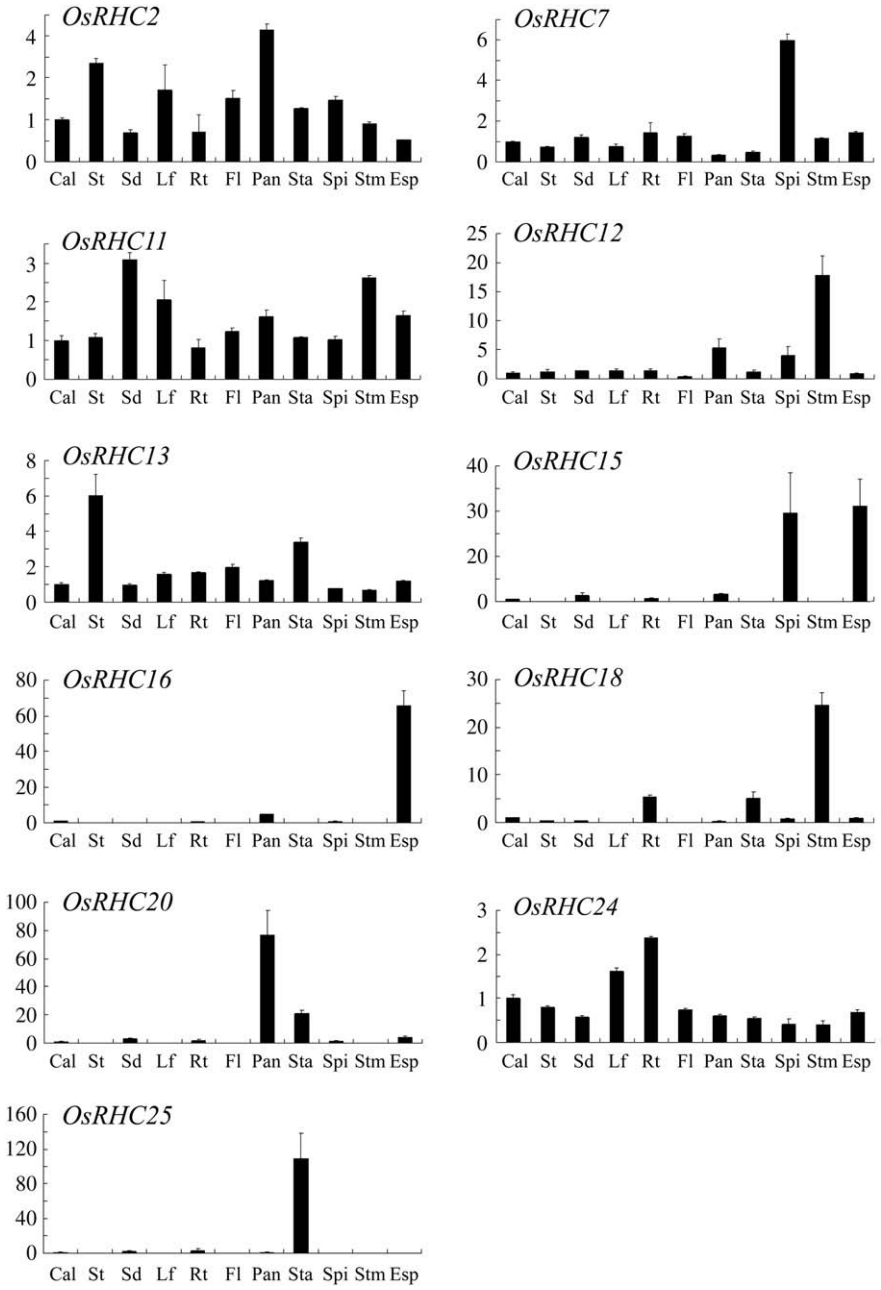

Fig. 7. Real-time PCR of genes showing tissue-preferential expression. The *x*-axes are representative tissues and *y*-axes are scales of relative expression level. Eleven representative tissues are as follows: Cal, callus at 15 days after subculture; St, young shoots (3 days after germination); Sd, three-leaf seedlings; Lf, leaf from plants with two tillers; Rt, root from plants with two tillers; Fl, leaf from plants with young panicle 4–5 cm in length; Pan, young panicle 4–5 cm in length; Spi, spikelet 3 days after pollination; Stm, stem from plants at 1 day before flowering; Stm, stamen at 1 day before flowering; Esp, endosperm at 7 days after pollination. The bars are standard deviations of three technical repeats.

heading stage, whereas *OsRHC20* was exclusively expressed in panicle of heading stage and stamen of 1 day before flowering. *OsRHC25* was exclusively expressed in panicle of heading stage. It seemed that these tissue- or organ-preferentially expressed genes were inclined to express in reproductive organs. In addition, six C3HC4-type RING finger genes (*OsRHC2*, *OsRHC7*, *OsRHC11*, *OsRHC12*, *OsRHC13* and *OsRHC25*) which had been studied before were also investigated. The relative expression level of these genes in all tissues compared with callus at 15 days after subculture was indicated in Fig. 7. The results showed that the expression level of *OsRHC2* was high in 4–5 cm young panicle (3.14 times) and shoot from seedling with 2 tillers (2.36 times), respectively. *OsRHC7* was highly expressed in spikelet of 3 days after pollination (6 times). The transcripts of *OsRHC11* were relatively high in three-leaf seedlings (3 times), leaf from plants with two tillers (2 times) and stamen at 1 day before flowering (2.6 times). It was worth mentioning that *OsRHC12* showed preferential expression in stamen (18 times). In addition, the expression level of *OsRHC13* in shoot from seedling with 2 tillers and in stamen of one day before flowering was 6 times and 3 times higher than that in callus, respectively. *OsRHC24* was moderately expressed in root of plants with two tillers (2.8 times).

3.6. Differential expression profiles of C3HC4-type RING finger family genes under hormone treatment

According to our microarray data, 12 C3HC4-type RING finger family genes (*OsRHC2*, *OsRHC3*, *OsRHC7*, *OsRHC11*, *OsRHC12*, *OsRHC13*, *OsRHC14*, *OsRHC16*, *OsRHC19*, *OsRHC22*, *OsRHC24* and *OsRHC28*) were differentially regulated by at least one hormone treatment. Real-time PCR analysis was performed to confirm these genes' expressions

under different hormone treatments (Fig. 8). The results showed that the expressions of all these 12 genes were affected by ABA, JA and BR treatments. The expressions of 4 genes (*OsRHC2*, *OsRHC13*, *OsRHC16* and *OsRHC22*) were influenced by all the 6 hormones used in this experiment. Two genes (*OsRHC16* and *OsRHC22*) were induced by all these 6 hormone treatments and the other two genes (*OsRHC2* and *OsRHC13*) were either up-regulated or down-regulated with these hormone treatments. Three genes (*OsRHC3*, *OsRHC14* and *OsRHC24*) were influenced by 5 hormone treatments and two genes (*OsRHC11* and *OsRHC12*) were influenced by 4 hormone treatments. The expressions of all the 12 genes were induced by ABA. Among the 12 genes, 6 genes (*OsRHC12*, *OsRHC14*, *OsRHC16*, *OsRHC22*, *OsRHC24* and *OsRHC28*) were induced by JA and BR while the remains were suppressed by JA and BR. Seven genes (*OsRHC2*, *OsRHC3*, *OsRHC13*, *OsRHC14*, *OsRHC16*, *OsRHC22* and *OsRHC24*) were influenced by IAA, 7 genes (*OsRHC2*, *OsRHC11*, *OsRHC12*, *OsRHC13*, *OsRHC16*, *OsRHC22* and *OsRHC24*) were regulated by GA_3, and 6 genes (*OsRHC2*, *OsRHC3*, *OsRHC13*, *OsRHC14*, *OsRHC16* and *OsRHC22*) responded to KT.

3.7. Expression profiles of the C3HC4-type RING finger family genes under different stresses

As all 12 genes mentioned above were induced by ABA, we speculated that these C3HC4-type RING finger genes may be involved in the signaling pathways triggered by abiotic stresses. To reveal the responses of these C3HC4-type RING finger genes to different stresses, real-time PCR analysis was performed using total RNA from the leaves of rice cultiva Zhonghua 11 treated with salt, drought, H_2O_2 and cold, respectively (Fig. 9). The results showed that among the 12 genes, 9 genes (*OsRHC7*, *OsRHC11*, *OsRHC12*, *OsRHC13*, *OsRHC14*, *OsRHC16*, *OsRHC22*, *OsRHC24* and *OsRHC28*) were induced, 2 genes (*OsRHC2* and *OsRHC3*) were suppressed and 1 gene was not changed (*OsRHC19*) by salt. 10 genes (*OsRHC2*, *OsRHC7*, *OsRHC12*, *OsRHC13*, *OsRHC14*, *OsRHC16*, *OsRHC19*, *OsRHC22*, *OsRHC24* and *OsRHC28*) were induced, 1 gene (*OsRHC11*) was suppressed and 1 gene (*OsRHC3*) was not influenced by drought. Seven genes (*OsRHC7*, *OsRHC12*, *OsRHC13*, *OsRHC14*, *OsRHC16*, *OsRHC19* and *OsRHC22*) were induced, one gene (*OsRHC11*) was suppressed and 4 genes (*OsRHC2*, *OsRHC3*, *OsRHC24* and *OsRHC28*) were not affected by H_2O_2. Three genes (*OsRHC13*, *OsRHC16* and *OsRHC22*) were induced, 7 genes (*OsRHC2*, *OsRHC3*, *OsRHC7*, *OsRHC14*, *OsRHC16*, *OsRHC19* and *OsRHC24*) were suppressed and 2 genes (*OsRHC11* and *OsRHC28*) were not affected by cold.

Our results also showed that these 12 C3HC4-type RING finger genes were responsive to more than one kind of stresses. For example, among the 9 salt-inducible genes, 9 genes were differentially regulated by drought, 6 by H_2O_2, and 6 by cold. Among the 10 drought-inducible genes, 9 genes were differentially influenced by salt, 8 by H_2O_2, and 8 by cold. Among the 7 H_2O_2-inducible genes, 6 genes were differentially regulated by salt, 7 by drought, and 7 by cold. Five genes (*OsRHC7*, *OsRHC12*, *OsRHC13*, *OsRHC14* and *OsRHC16*) were responsive to all four stresses. We also noticed that most genes were stress-inducible except 5 genes: *OsRHC2* and *OsRHC3* were suppressed by salt and cold; *OsRHC7* and *OsRHC19* were suppressed by cold; *OsRHC11* was suppressed by drought and H_2O_2.

4. Discussion

4.1. C3HC4-type RING finger proteins possess various functions in diverse physiologic process

Previous reports revealed that C3HC4-type RING finger proteins play different roles in diverse physiologic processes. It has been proven that most C3HC4-type RING finger proteins possess E3 ubiquitin ligase activity. Ubiquitin-mediated protein modification regulates many cellular processes, including homeostasis, development, cell division, growth, and hormone and stress responses (Smalle and Vierstra, 2004). Since the specificity of the ubiquitin proteasome pathway is determined by E3 ligase, the various functions of C3HC4-type RING fingers may be explained by the fact that they act as E3 ligase on different targets in diverse physiologic processes. In this study, the differentially expressed patterns and responses to stress conditions and chemical treatments also imply the different biologic functions of the members in this gene family.

4.2. Implication of the cis-elements for functions of the C3HC4-type RING finger genes

The *cis*-elements play key roles in the transcriptional regulation of genes controlling various biologic processes including abiotic stress responses. Several types of *cis*-elements have been identified and analyzed for their activation by different stresses, such as ABA-responsive element (ABRE), dehydration-responsive element (DRE) and low-temperature-responsive element (LTRE), which have been extensively characterized for their important roles in activation of gene expression under stress conditions (Shinwari et al., 1998; Shinozaki and Yamaguchi-Shinozaki, 2000; Narusaka et al., 2003). All these *cis*-elements have also been identified in this study. The most enriched stress-related *cis*-elements identified in rice C3HC4-type RING finger gene family are MYBCORE and GT1GMSCAM4. MYBCORE is a *cis*-element that functions in dehydration, which is a major physiologic response when plant cells are exposed to low temperature, drought, and high salinity. GT1GMSCAM4 is a type of *cis*-element related to salt, which interacts with a GT-1-like transcription factor that plays a role in pathogen- and salt-induced gene expression in both soybean and *Arabidopsis* (Park et al., 2004). In addition, the ABA-responsive elements, EBOXBNNAPA and MYCCONSENSUSAT, were also widely distributed in the rice C3HC4-type RING finger genes. ABA is the primary hormone that mediates plant responses to stresses such as cold, drought and salinity (Sheen, 1996; Shinozaki and Yamaguchi-Shinozaki, 2000). These results suggested that these C3HC4-type RING finger genes may plant important roles in regulating stress responses.

We also found some *cis*-elements related to protein storage, RNA processing, calcium signaling and light responses. SEF4MOTIFGM7S is responsive to protein storage and CGCGBOXAT is a binding site for calmodulin-binding protein. GT1CONSENSUS (GRWAAW) is the binding site of the nuclear regulatory protein GT-1 (Nagano, 2000). The wide distribution of GT1CONSENSUS is related to the function of light for regulating many physiologic and biochemical processes in growth and development. The existence of these *cis*-elements in rice C3HC4-type RING finger genes and their key roles in regulating gene expression suggested that C3HC4-type RING finger genes may participate not only in stress responses but also in different developmental processes in rice.

4.3. Hormonal regulation of C3HC4-type RING finger genes

Phytohormone is the most important signaling molecule in plants. The concentration of the phytohormone influences the growth and development of the plants. Auxin regulates cell processes and especially promotes cell elongation. GAs are a large family of plant hormones, some of which are bioactive growth regulators, controlling seed germination, stem elongation, and flowering (Yamaguchi, 2008). KTs are compounds that stimulate cell division or cytokinesis. Brassinosteroids are steroidal plant hormones which play their roles in the promotion of plant growth and development (Bajguz and Hayat, 2009). Jasmonates (JAs) are signaling molecules that play a key role in regulation of metabolic processes, reproduction, and defense against pathogens and insects (Balbi and Devoto, 2008).

Previous studies have indicated that the ubiquitin proteasome system (UPS) is an integral part of multiple hormone-signaling pathways (Dreher and Callis, 2007). The UPS allows plants to effectively and efficiently alter their proteasome to ensure developmental

plasticity and environmental adaptation. In response to variations in hormone levels, the UPS regulates the abundance of signaling factors, mainly hormone-responsive transcription factors, which mediate cellular responses. Recent exciting studies have shown that hormones directly or indirectly modulate substrate ubiquitination by regulating E3-substrate interaction (Stone and Callis, 2007).

Previous studies have shown that overexpression of the RING finger gene *AtSDIR1* leads to ABA hypersensitivity and ABA-associated phenotypes, such as salt hypersensitivity in germination, enhanced ABA-induced stomatal closing, and enhanced drought tolerance (Zhang et al., 2007). Another C3HC4 RING finger gene *OsCOIN1* (*OsRHC13*) is expressed in all rice organs and strongly induced by ABA (Liu et al., 2007a). In addition, *AtXBAT32* is induced by auxin and the lateral root defect in *xbat32-1* mutant plants could be rescued under auxin treatment. Thus, AtXBAT32 is a novel ubiquitin ligase required for lateral root initiation (Nodzon et al., 2004). Our results showed that the 12 rice C3HC4 RING finger genes were regulated by hormones, such that all these 12 genes were affected by ABA, JA and BR, 7 genes were influenced by IAA, 7 genes were regulated by GA_3, and 6 genes responded to KT. These results suggested that these C3HC4-type RING finger proteins may be involved in hormone-signaling pathways through the UPS.

4.4. C3HC4-type RING finger genes play an important role in the stress response

ABA has been considered as an important “stress hormone” as it plays a critical role in plants' response to various stresses. The application of ABA to plants partially mimics the effect of stress conditions (Nambara and Marion-Poll, 2005). It is well known that ABA

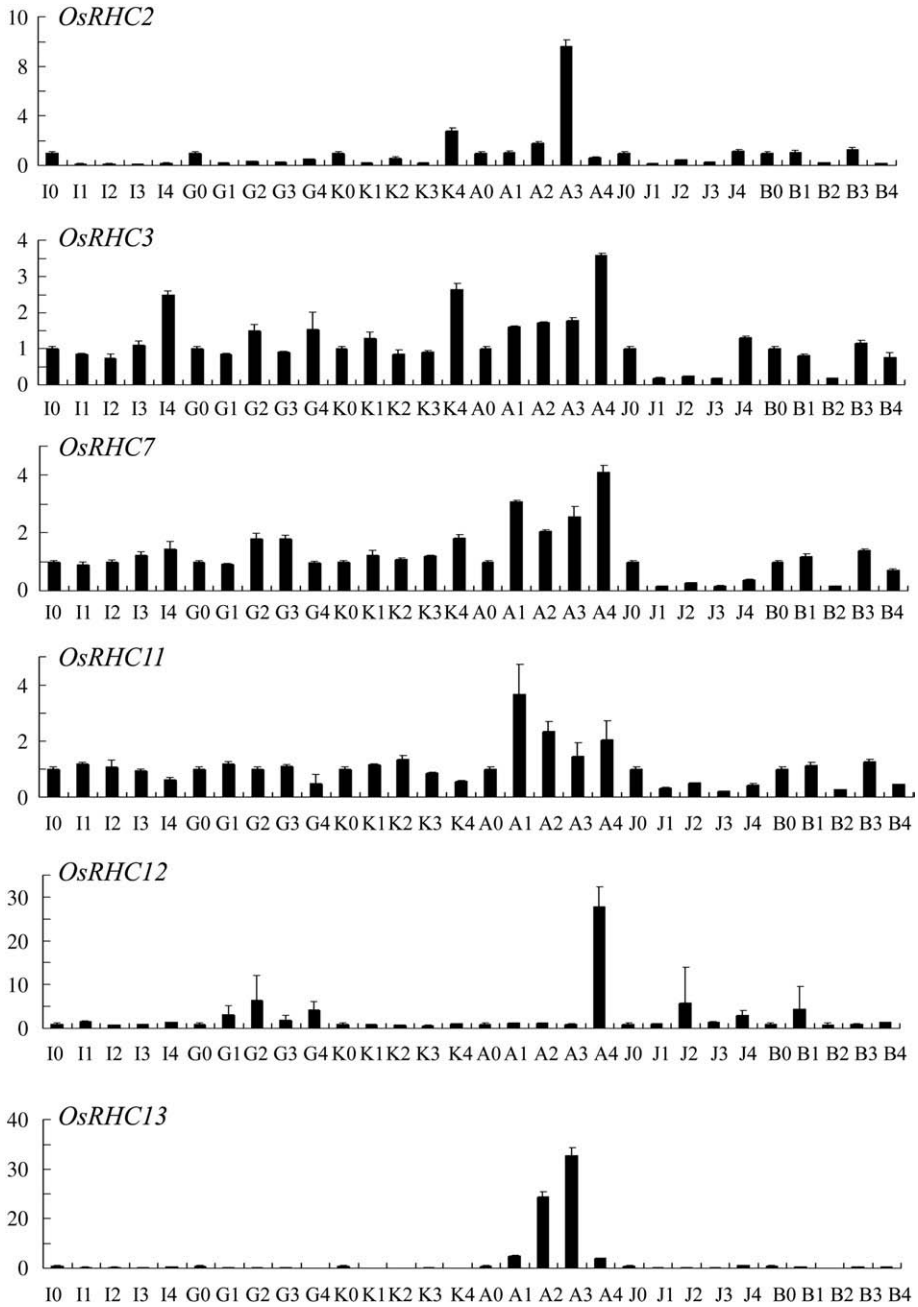

Fig. 8. Real-time PCR analysis of hormone-responsive C3HC4-type RING finger genes. The *x*-axes are time courses of hormonal stress treatments and *y*-axes are scales of relative expression level. I, IAA; G, GA3; K, KT; A, ABA; J, JA; B, BR. For each hormone treatment, rice leaves were sampled at 0, 1, 3, 6, and 12 h after treatment, respectively. The bars are standard deviations of technical repeats.

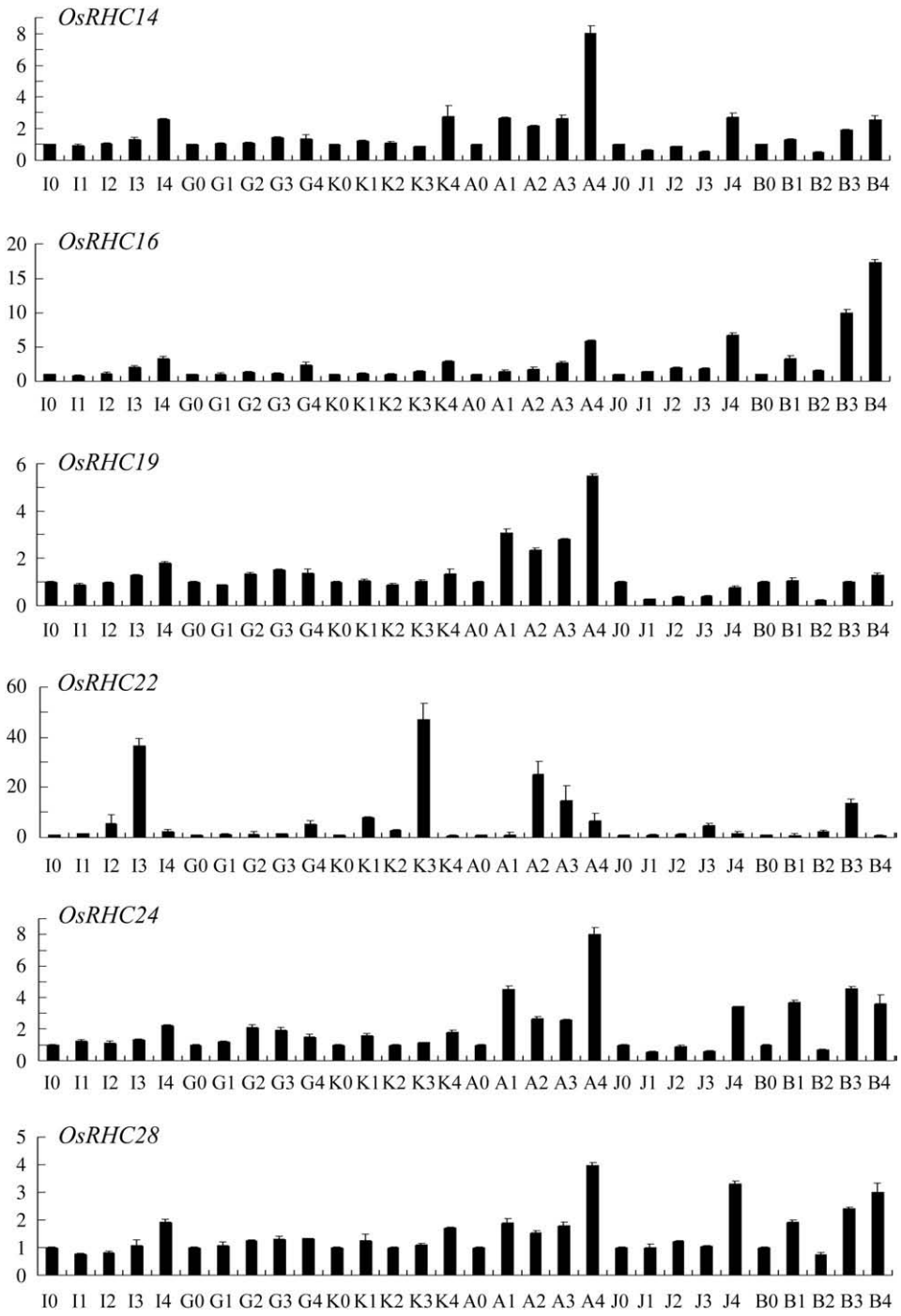

Fig. 8 (*continued*).

modulates gene expression under osmotic stresses such as freezing, drought, and salt (Jensen et al., 1998). Both ABA-independent and ABA-dependent signal transduction pathways have been suggested to participate in the water stress response and stress-responsive gene expression (Park et al., 2004). In this study we found that 12 of the 29 C3HC4-type RING finger genes are induced by ABA and each of the 12 C3HC4-type RING finger genes was differentially regulated by multiple stresses. Most genes were induced by salt, drought and H_2O_2 and suppressed by cold. These results indicate that C3HC4-type RING finger genes may be involved in the substantial common regulatory systems or cross-talks triggered by different stresses. Because many abiotic stresses ultimately result in dehydration and osmotic imbalance of plant cells, there is a large overlap of genes induced by drought and salt stresses (Lee et al., 2001; Ludwig et al., 2005). Based on the stress-induced expression patterns of C3HC4-type RING finger genes, our results also support the notion of cross-talks between drought and salt stresses (Seki et al., 2002a, 2002b). In addition, our results also suggested that the responsiveness of these C3HC4-type RING finger genes to abiotic stresses may be mainly mediated by ABA-dependent pathways.

4.5. Preferential expression of C3HC4-type RING finger genes in reproductive tissues or organs

Usually a high or preferential expression in tissues or organs suggests that the genes may play an important role there. In this study we

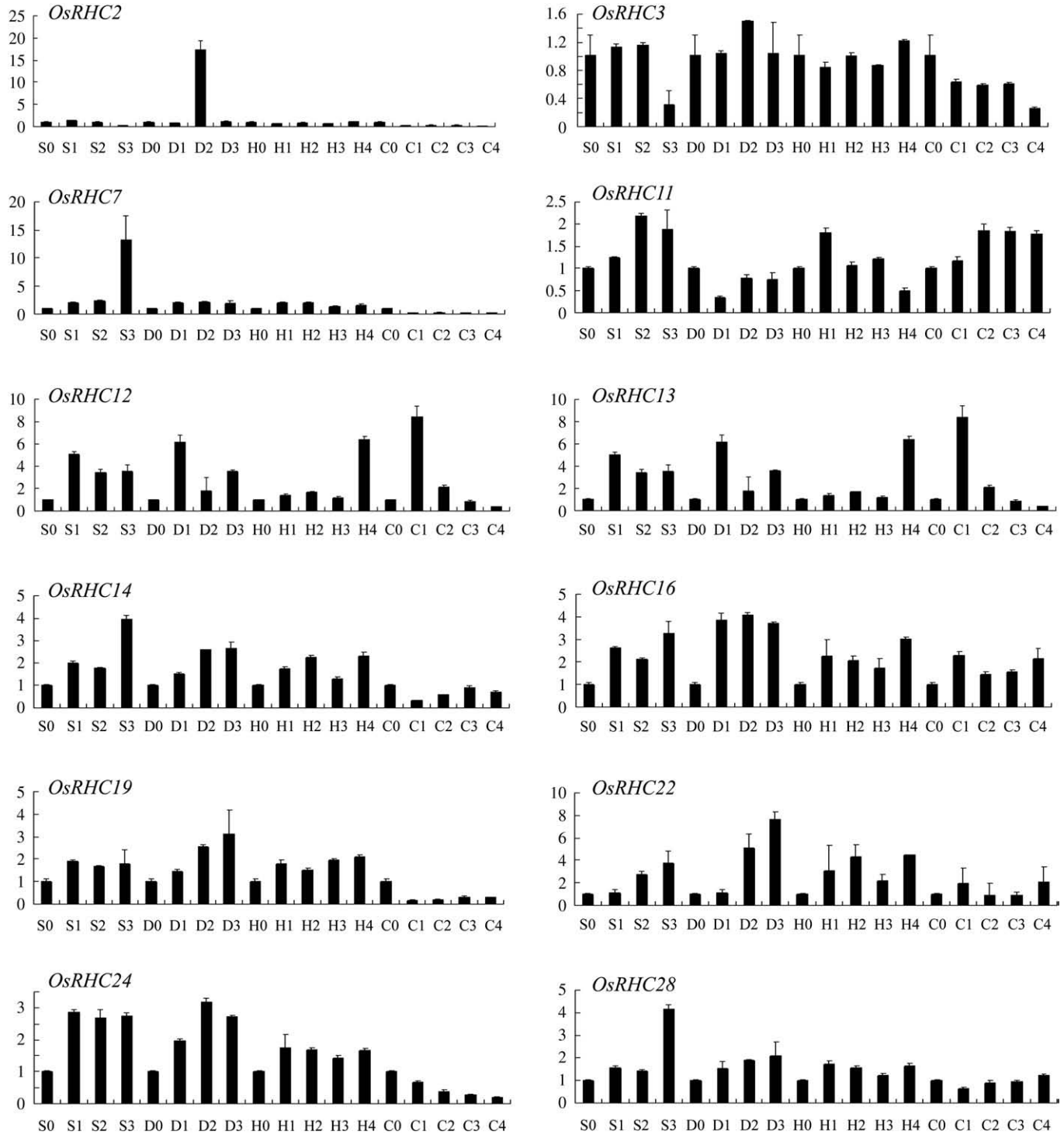

Fig. 9. Real-time PCR analysis of stress-responsive C3HC4-type RING finger genes. The *x*-axes are time courses of abiotic stress treatments and *y*-axes are scales of relative expression level. S, salt; D, drought; H, H_2O_2; C, cold. For salt stress and drought stress, seedlings were sampled at 0, 1, 6, and 12 h, respectively. For H_2O_2 and cold stress, seedlings were sampled at 0, 1, 3, 6, and 12 h, respectively. The bars are standard deviations of technical repeats.

found that among 29 C3HC4-type RING finger genes, only 5 genes showed tissue-preferential expression patterns. The most interesting thing was that the 5 genes were preferentially expressed in reproductive tissues or organs, indicating that they may play an important role at the reproductive stage. These tissue- or organ-preferentially expressed genes may deserve special notice for further investigation on their functions because many C3HC4-type RING finger genes in plants have been proven to play important roles in regulating growth and development.

4.6. Conclusion

Taken together, this study provides not only an annotation of the C3HC4-type RING finger family in rice, but also the identification of many tissue-preferentially expressed, hormone-responsive or stress-responsive genes. These data provide important information for insights into the functions of C3HC4-type RING finger family genes and may contribute to the genetic improvement of rice.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.gene.2009.05.018.

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