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Systematic identification of *X1*-homologous genes reveals a family involved in stress responses in rice

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Abstract *X1*-homologous genes (*XHS*) encode plant-specific proteins containing three major domains (XH, XS, zf-XS), but their functions are largely unknown. We report the systematic identification and characterization of *XHS* genes in the rice genome. Eleven putative XHS protein sequences (OXHS1–11) were identified in the sequenced genome of *Oryza sativa japonica* cv. Nipponbare, and these sequences, along with other plant XHS homologues, were classified into five subgroups based on phylogenetic analysis. Distinct diversification of the XHS proteins occurred between monocotyledon and dicotyledon plants. The OXHS family has diverse exon–intron structures and organizations of putative domains and motifs. The OXHS proteins showed no transactivation activity, and no interaction between the XH domain and the XS domain in yeast. Four representative OXHS proteins were targeted to cytoplasm, which contradicts the previous speculation that XHS proteins are putative transcription factors. All the *OXHS* genes are predominantly expressed in floral organs, and some are expressed in a wide range of tissues or organs in *indica* rice Minghui 63. Nine *OXHS* genes are responsive to at least one of the abiotic stresses including drought, salt, cold, and abscisic acid treatment. Over-expression of one stress-responsive gene *OXHS2* in rice resulted in reduced tolerance to salt and drought stresses. These results suggest that the OXHS family may be functionally diversified and some members of this family may play important roles in regulating stress tolerance in rice.

Keywords Oryza · XHS family · Abiotic stress · Over-expression

Abbreviations

ABA	Abscisic acid
XH	X1 homologue
XHS	XH and XS domain
PCR	Polymerase chain reaction
RT	Reverse transcription

Introduction

Sessile plants have evolved numerous plant-specific genes for growth and development and responses to the ever-changing environment across their life cycles. Although the genomes of plants such as *Arabidopsis* and rice have been finely sequenced and intensively annotated on the basis of bioinformatics analysis, the biological functions of a large proportion of the genes in these genomes remain to be experimentally elucidated. This is especially true for many plant-specific gene families that are potentially important for both basic biological processes, such as growth and development, and plant-specific responses and adaptation to environmental stresses. Detailed analyses of developmental and phenotypic changes of transgenic or mutant plants and spatio-temporal expression patterns of some genes have greatly contributed to our understanding of

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plant architecture and elucidation of the developmental programs of the life cycle (Itoh et al. 2005). In addition, it is of fundamental biological importance to understand the functions of the numerous unknown genes for perceiving environmental signals and transmitting the signals to cellular machinery to activate adaptive responses (Mittler 2006).

Increasing evidence suggested that some gene families have very significant roles both in development and response to stress. Examples are the genes from the C-terminal domain phosphatase-like (CPL) and F-BOX families and many transcription factor families such as NAC and MYB. The CPL family is a focal control point of complex processes such as plant stress responses and development with unique and overlapping transcriptional regulatory functions that differentiate the signal output that determines the plant response (Koiwa et al. 2002). F-BOX family genes are required for panicle, leaf, and seed development (Chae et al. 2004; Durfee et al. 2003; Woo et al. 2001) and are regulated by light and temperature stress (Calderon-Villalobos et al. 2007; Jain et al. 2007). NAC transcription factor family genes are involved in the controls of lateral root development (Xie et al. 2000) and the formation of secondary walls in woody tissues in *Arabidopsis* (Mitsuda et al. 2007; Yamaguchi et al. 2008) and drought and salt stress resistance (He et al. 2005; Hegedus et al. 2003; Hu et al. 2006). MYB family genes have important roles in the formation of root hairs, flower development, and stress responses. For example, the MYB gene family has pleiotropic effects on flower development, epidermal cell size, and trichome branching in addition to trichome and root hair formation in *Arabidopsis thaliana* (Rotman et al. 2005; Tominaga et al. 2008; Zhu et al. 2004), and many genes of this family also participate in the regulation of freezing, drought, and salt stress tolerance (Dai et al. 2007; Urao et al. 1993).

Gene *X1* was first identified in rice, and this gene encodes a protein with putative protein–protein interaction and zinc finger domains (Chen and Bennetzen 1996). The first *X1*-homologous gene in *Arabidopsis* was *SGS3*, which was identified through analysis of the *SGS3* mutant impaired in post-transcriptional gene silencing (Mourrain et al. 2000). Through a detailed analysis of the homologous sequences of SGS3, a plant-specific gene family with unknown function was identified and the genes of this family were predicted to encode putative transcription factors (Bateman 2002; Mourrain et al. 2000).However, no experimental evidence has been reported so far to support the speculation that the family members are transcription factors. The predicted protein sequences of *X1*- or *SGS3*-homologous genes contain one to three of three novel protein domains: XH, XS, and zf-XS (Bateman 2002), and this family was thus named XHS family. The XS domain contains a completely conserved aspartate and a predicted secondary structure with mixed alpha helix and beta sheet. The XH domain is located at the C-terminal region of XHS proteins and contains one completely conserved glutamate that may be a functionally important site. The zf-XS domain, which usually accompanies a XS domain, is an N-terminal cysteine/histidine zinc binding domain (Bateman 2002).To date, no experimental data have been reported regarding the functions of this family, except for the member SGS3 in *Arabidopsis*. SGS3 is required for post-transcriptional gene silencing and natural virus resistance (Mourrain et al. 2000), which are needed for endogenous gene silencing from DNA viruses (Glick et al. 2008; Muangsan et al. 2004) and for juvenile development and the production of trans-acting siRNAs in *Arabidopsis* (Peragine et al. 2004).

Although the sequence characteristics of typical XHS proteins were well addressed (Bateman 2002), no systematic identification and functional insight of this family in a given plant species had been reported. Here, we analyzed the sequences of genes containing XH, XS, and/or zf-XS domains in rice (designated as the *OXHS* family) and investigated the expression profiles of this family in various tissue or organs and under stress treatments. In addition, we investigated the phenotypic changes of the mutants and/or over-expression transgenic plants of a few genes in relation to development or response to abiotic stresses. Our results suggested that the XHS family may play important roles in response to environmental stresses.

Materials and methods

Identification and sequence analysis of OXHS family

Using the BLAST program, the protein sequences of known rice protein *X1* (Q9SBW2) and *Arabidopsis* protein SGS3 (Q9DLX1) from the protein sequence database ExPASy (http://www.expasy.org/) (Gasteiger et al. 2003) were used to search for homologous proteins in the databases of *japonica* rice (TIGR: http://www.tigr.org, Yuan et al. 2005; KOME: http://cdna01.dna.affrc.go.jp/cDNA, Kikuchi et al. 2003) and of *Arabidopsis* (TAIR: http://www.arabidopsis.org, Huala et al. 2001). The pHMM was used to identify new XHS sequences in the rice and *Arabidopsis* genomes. The HMM profile of XH domain (accession no. PF03469), XS domain (accession no. PF03468), and zf-XS domain (accession no. PF03470) were downloaded from the Pfam database (http://www.sanger.ac.uk/Software/Pfam, Bateman et al. 2004). All hits with expected values less than 1.0 were collected. We used a BLASTN search to determine the chromosome locations of *OXHS* genes, the rice BAC/PAC clones to which they

were mapped, and the intron–exon structures of *OXHS* genes by mapping cDNAs to genomic sequence.

Multiple sequence alignment of XHS proteins was performed with CLUSTALX (van de Graaff et al. 1982) and refined manually. Phylogenetic tree was reconstructed with the program MrBayes version 3.0 (Ronquist and Huelsenbeck 2003) under the JTT-f model of amino acid substitution and 4-g category model. A total of 400,001 generations were performed with four Markov chains with default heating values and tree sampling every 100 generations. The Markov chain converged after 9,000 generations, and the first 100 sampled trees were discarded. The major consensus tree was deduced from the remaining 901 sampled trees. The tree was edited with TreeView 1.5 (Page 1996).

Isolation of XHS genes and rice transformation

For each *OXHS* gene, a pair of primers (Supplemental Table 1) was designed to amplify the predicted full-length cDNA from different tissues of Minghui 63 (*Oryza sativa* L. ssp. *indica*). Ex-Taq DNA polymerase (Takara) was used in the PCR with the following cycling profile: 94°C for 4 min; 30 cycles of 94°C for 40 s, 55°C for 40 s, and 72°C for 2 or 3 min; and extension at 72°C for 10 min. The amplified products were cloned into pGEM-T vector (Promega) and sequenced from both ends by using BigDye Terminator Sequencing Ready kit (version 2.0 or 3.0) in an ABI PRISM 377 or 3730 sequencer (Applied Biosystems) by the vector-border primers T7 and SP6 (Promega). The sequence-confirmed fragments of *OXHS* genes were cut by *Kpn*I and/or *Bam*HI from pGEM-T clones and ligated into the transformation vector pCAMBIA1301U under the control of the promoter of maize ubiquitin gene. The *Agrobacterium*-mediated transformation method was used to introduce the constructs into rice Zhonghua 11 (*Oryza sativa* L. ssp. *japonica*) (Hiei et al. 1994).

Plant growth, treatments, and measurements

For transcript level measurement of *OXHS* genes, rice plants of Minghui 63 were grown under normal growth conditions and 2-week-old seedlings were treated with ABA and abiotic stresses. ABA treatment was conducted by spraying leaves with 0.1 mM followed by sampling at 0, 0.5, 2, 4, and 8 h. Abiotic treatments were conducted essentially according to Saijo (2000). For cold stress, seedlings were transferred to a growth chamber at 4°C and sampled at 0, 0.5, 1, 3, and 6 h after treatment. Drought stress at the seedling stage was applied by exposing 3-week-old intact plants to the air without water supply and plant leaves were sampled at 0, 0.5, 2, 4, and 8 h after treatment. The seedlings were submerged with 200 mM NaCl solution for salt stress and sampled at 0, 15 min, 30 min, 1 h, and 2 h after treatment. For salt or mannitol resistance testing of plants at the seedling stage, the plantlets that germinated on plates with MS medium were transferred to MS medium with 150 mM NaCl or mannitol in autoclaved visible boxes. The lengths of shoot and fresh weight were measured 1 week after transplanting. Free proline content in leaves was determined by following the methods reported by Ohnishi et al. (2005).

Drought stress was applied to transgenic plants growing in field at the panicle development stage. Sixteen plants were grown in two rows for each transgenic family with three replications. The wild type (WT) control was inserted every two transgenic families to minimize the potential variation of soil the field. Relative spikelet fertility or yield, the ratio of the spikelet fertility or yield under stress conditions to that under normal growth conditions, was used to evaluate drought resistance performance at the reproductive stage of rice according to Yue et al. (2006). Drought stress treatment, data collection and statistical analysis were conducted according to Xiao et al. (2009).

Transcript level analysis

Total RNA was extracted from various tissues and organs from the life cycle of Minghui 63 using the TRIZol reagent (Invitrogen) according to the manufacturer's instructions. The total RNA was treated with amplification-grade DNase I (Invitrogen) for 15 min to degrade possibly contaminated residual genomic DNA. SuperScript II reverse transcriptase (Invitrogen) was used to synthesize first-strand cDNA and about 1/20 of the first-strand cDNA generated from 3 ug total RNA was used as template for PCR in a reaction volume of 50µl with the rTaq DNA polymerase (Takara). Semi-quantitative PCR or RT-PCR was performed in an ABI 9700 Thermocycler (Applied Biosystems) with the following cycling profile: 94°C for 3 min; 25–40 cycles (depending on the expression level) at 94°C for 40 s, 55°C or 60°C for 40 s, and 72°C for 1 min. The RT-PCR product was separated in a 1.2% agarose gel and stained with ethidium bromide for visualization. We used rice *Actin1* gene (accession no. AK060893) as the internal control. For each *OXHS* gene, a pair of primers with an average amplification length of 500 bp was used for the RT-PCR. All experiments were repeated three times with independently reverse-transcribed templates.

Relative quantification of gene expression was performed by real-time PCR on an ABI PRISM 7500 instrument (Applied Biosystems). The primers for real-time PCR were designed by Primer Express Version 2.0 (Applied Biosystems; Supplemental Table 2). Rice *Actin1* gene was used as the endogenous control. Real-time PCR was

performed in an optical 96-well plate, including 12.5 μl 23 SYBR Green Master mix reagent (Applied Biosystems), 1 μl cDNA samples, and 0.2 mM of each gene-specific primers, in a final volume of 25 μl, using the thermal cycles as follows: 50°C for 2 min, 95°C for 10 min, 40–45 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 1 min. Disassociation curve analysis was performed as follows: 95°C for 15 s, 60°C for 20 s, 95°C for 15 s. The relative expression levels were determined as described previously.

Biochemical assay in yeast

For yeast assay, the full ORFs of *OXHS1*, *OXHS2, OXHS8,* and *OXHS10* were generated by PCR using the primer pairs (Supplemental Table 3) and fused in frame to the yeast GAL4 DNA-binding domain in PDBLeu vector by restriction-ligation reaction or in pDESTT32 by Gateway recombination reaction. The full ORFs were fused to the yeast GAL4 DNA-activation domain in AD502 or in pDESTT22. For transactivation assay, fusion constructs of OXHS-GAL4 DNA-binding domain were transformed in yeast strain MV203. For protein–protein interactions, one fusion protein of OXHS-GAL4 DNA-binding domain and another fusion protein of OXHS-GAL4 activation domain were co-transformed into the yeast strain MV203. The colony-lift filter assay (X-gal assay) was performed to check the transactivation activity or protein–protein interaction as described by the manufacturer (Invitrogen).

To determine the subcellular localization of the OXHS proteins, the coding regions of *OXHS* gene amplified using the primer pairs (Supplemental Table 3) was fused in frame to the coding sequence of enhanced green fluorescence protein (EGFP) under the control of a maize ubiquitin promoter in the vector of pCAMBIA1391Xb. The constructs were introduced into onion (*Allium cepa*) epidermal cells by the bombarding method and fluorescence signal was captured by confocal microscopy.

Results

Identification of XHS family genes in rice and *Arabidopsis*

To identify all putative genes containing the XH-XS domain in rice, two reported protein sequences containing the XH-XS domain (X1 and SGS3) were used as queries to search the GeneBank database, the rice annotation database at The Institute for Genomic Research (TIGR), and the Knowledge-Based Oryza Molecular Biological Encyclopedia (KOME). After removing the redundant sequences, a total of 11 genes encoding XH-XS-domain-containing proteins, designated as *OXHS1–OXHS11* (Table 1), were identified in the *japonica* rice genome. Some generic information for these *OXHS* genes are listed in Table 1, including the accession numbers of full-length cDNAs available in KOME and corresponding BAC/PAC clones and their chromosome locations. In the KOME database, the full-length cDNAs were available for all *OXHS* genes except for *OXHS7, OXHS9*, and *OXHS11*. Based on a comparison of the full-length cDNAs and the corresponding genomic sequences for the *OXHS* genes in GenBank or TIGR, we noticed that the 5′-terminus of the open reading frame (ORF) of *OXHS2* (cDNA accession no. AK065750) was annotated incorrectly in KOME, and thus a portion of the XS domain in the predicted protein sequence of OXHS2 in TIGR (LOC_Os01g03570) is absent in the predicted protein sequence of the cDNA. Therefore, we isolated the full-length cDNA of this gene again from *Oryza sativa japonica* cv. Nipponbare and confirmed that the annotation based on the genomic sequence in TIGR database is correct (not shown). The cDNA sequences for complete ORFs of all the *OXHS* genes except *OXHS11* were also successfully isolated from *O. sativa indica* cv. Minghui 63 with gene-specific primers (Supplemental Table 1). Comparison of the cDNA sequences of *OXHS* genes from Minghui 63 and Nipponbare revealed a few single-nucleotide polymorphisms in four *OXHS* genes that led to a few changes of amino acids (Supplemental Table 4). However, all the amino acid changes occurred in nonconserved regions, except one change in the XH domain of *OXHS5*. For simplicity, all the subsequent analyses were based on the sequences of *OXHS* genes from the *japonica* rice Nipponbare.

Table 1 General information about *OXHS* family genes

Gene name	Locus[a]	BAC[b]	Accession no of cDNA
OXHS1	LOC_Os01g44230	AP004317	AK069077
OXHS2	LOC_Os01g03570	AP002909	AK065750
OXHS3	LOC_Os05g06130	AC093493	AK063522
OXHS4	LOC_Os02g19130	AP003988	AK242745
OXHS5	LOC_Os03g39910	AC120537	AK107535
OXHS6	LOC_Os01g05470	AP002872	AK066686
OXHS7	LOC_Os01g05440	AP002872	
OXHS8	LOC_Os03g06340	AC099401	AK060572
OXHS9	LOC_Os04g21730	AL662995	
OXHS10	LOC_Os12g38440	AL844878	AK064511
OXHS11	LOC_Os03g39940	AC120537	

[a] Accession number of *OXHS* genes in TIGR database

[b] Accession number of rice (*O. sativa* L. subsp. *japonica*) BAC clone

Using a similar search against the annotation database of *Arabidopsis* at The Arabidopsis Information Resource (TAIR), 14 genes encoding XH-XS-domain-containing

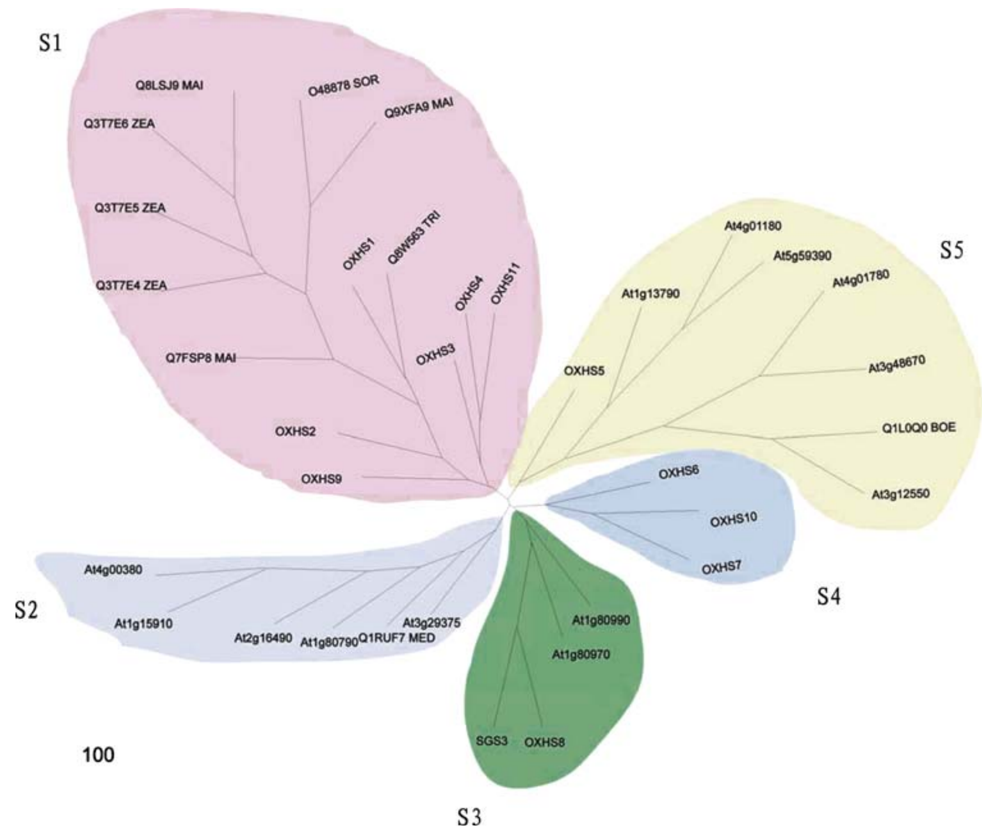

Fig. 1 Phylogenetic tree of XHS proteins in plants. Phylogenetic analysis was based on the full XHS proteins sequences of putative 35 XHS proteins from different plants including *Oryza sativa*, *Arabidopsis thaliana*, *Boechera drummondii, Sorghum bicolor, Medicago truncatula, Triticum monococcum,* and *Zea mays*

proteins were identified (Supplemental Fig. 1). Homologous sequences of XHS proteins were also found in other plant species but not in human and other animals, suggesting that XHS family may have evolved specifically in plants.

Phylogenetic analysis of XHS family

To study the evolutionary relationships of XHS family genes in plants, 35 putative XHS protein sequences, including 11 from rice, 14 from *Arabidopsis*, and 10 from other flowering plants, were collected for phylogenetic analysis. The results indicate that the XHS family is evolutionarily diversified. The collected XHS protein sequences can be classified into five subgroups (S1–S5) in the unrooted phylogenetic tree (Fig. 1). Nearly all XHS proteins from monocotyledon species (e.g., rice, maize, and wheat) are in subgroups S1 and S4, except that OXHS5 and OXHS3 are grouped into S5 and S3, respectively. Interestingly, all XHS proteins from dicotyledon plants (e.g., *Arabidopsis* and *Medicago truncatula*) are in subgroups S2, S3, and S5. Subgroup S4 contained only three rice proteins. These results suggest that plant XHS genes are derived from a common ancestor but differentiated separately in monocotyledon and dicotyledon plants. It is noteworthy that OXHS8 in subgroup S3 is the closest homologue to At5g23570, which is known as the SGS3 protein required for post-transcriptional gene silencing and natural virus resistance (Mourrain et al. 2000). Moreover, both OXHS8 and SGS3 contain only the XS domain, and not the XH domain present in most of the other XHS proteins. Such a strong similarity in both sequence and domain composition suggest that OXHS8 might be an orthologue of SGS3 in rice.

Diverse domain composition and gene structure in OXHS family

The OXHS protein sequences were further analyzed for putative domain compositions. Based on a Pfam database search, three putative domains including XH (PF03469), XS (PF03468), and zf-XS (PF03470) that have been identified in the X1 and SGS3 proteins were detected in the OXHS family. The 11 OXHS proteins can be classified into three types according to the composition of the putative domains detected (Fig. 2). The protein sequences in type I, including OXHS1, OXHS2, OXHS3, OXHS4, and OXHS6, contain the XH domain, XS domain, and zf-XS domain (except OXHS2). Four OXHS proteins (OXHS5,

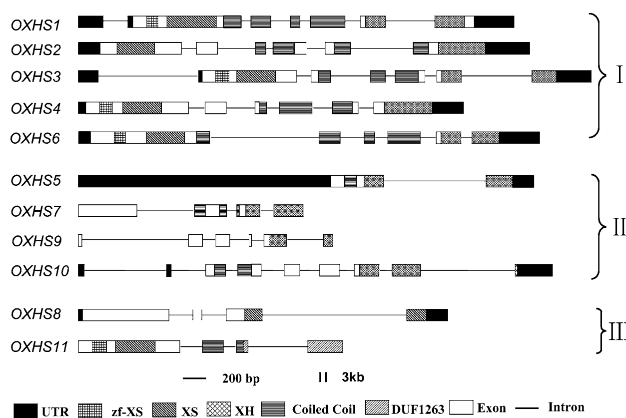

Fig. 2 Diverse domain composition and gene structure in the OXHS family. The *boxes* and *lines* represent exons and introns, respectively. The *double slash* (//) and *line bar* represent 3 kb and 200 bp, respectively. In the boxes, the *black regions* are UTR regions and *other regions* represent different putative domains as illustrated. The OXHS family can be classified into three types (I–III) based on the domain compositions

OXHS7, OXHS9, and OXHS10) each containing only the XH domain were classified as type II. Type III OXHS proteins (OXHS8 and OXHS11) contain only the XS domain, although OXHS11 has an additional zf-XS domain.

Sequence alignment of the putative domains of the XHS family in rice (Fig. 3) and *Arabidopsis* (Supplemental Fig. 2) revealed a few highly conserved amino acids or motifs in each domain. Among the three domains, zf-XS is the most conserved according to the sequence alignment. The zf-XS domain contains two highly conserved cysteine and histidine residues and is a C_2H_2 type zinc finger. In addition, some conserved leucine and arginine residues are also important to the zf-XS domain, which has been supposed to bind to the nucleic acid region (Bateman 2002). Except for SGS3 and OXHS8, the other XS domain proteins are highly conserved, especially for a completely conserved aspartate that was suggested to be an enzymatically active site (Bateman 2002). In addition to one completely conserved glutamate in the C-terminal region that could be part of an active site or other functionally important region, the XH domain also contains many conserved glutamate, aspartate, and proline residues, which may suggest that the XH domain is an activation domain with enriched acidic amino acids or proline, although these amino acids may not necessarily be important for function (Schwechheimer et al. 1998). The lengths of the zf-XS (50 AA), XS (110–120 AA), and XH (130–140 AA) domains are also quite conserved among most of the XHS proteins in rice and *Arabidopsis*. Nevertheless, OXHS8 and OXHS9 have a shortened XS (73 AA) and XH (78 AA) domain, respectively (Fig. 3). In addition, the exon–intron structures of the *OXHS* genes and splicing patterns had particular associations with the domain compositions of OXHS proteins. For example, the genes for type I proteins have six or seven exons with very similar splicing patterns and lengths of corresponding exons (Fig. 2).

Transactivation and subcellular location assays of OXHS proteins

The OXHS family had yet to be functionally characterized. The first reported *OXHS* gene (*X1*) was suggested to encode a transcription factor (Chen and Bennetzen 1996), although Bateman (2002) was suspicious of this inference. Meanwhile, Bateman (2002) predicted that the XS and XH domains might interact. Thus, far, however, there was no experimental support for these speculations.

To address the potential functions of OXHS proteins, we selected three proteins (OXHS1, OXHS8, and OXHS10, representing the three types of domain compositions shown in Fig. 2) to check if they have transactivation activity. The transactivation assay was performed in the yeast strain MV203 transformed with constructs of the full-length sequences of the three genes fused to the DNA-binding domain of GAL4 and the reporter construct corresponding to GAL4. None of the three OXHS proteins showed transactivation activity in the yeast strain (data not shown). Yeast two-hybrid assay suggested that none of the three selected proteins interacted with the other two proteins, and the XH or XS domain alone did not interact with the other domain or itself either (data not shown).

Most plant transcription factors contain a nuclear localization signal (NLS) characterized by a core peptide enriched in arginine (R) and lysine (K; (Boulikas 1994). By using the PredictNLS tool (http://www.predictprotein.org/cgi/var/nair/resonline.pl), we found that only OXHS1 had one potential NLS sequence with 47 amino acids (KKKKDFNINNLIQHASGVGAASNRQAKDK) in the N terminal. The subcellular locations of the OXHS proteins were predicted by MultiLoc (http://www.osc.edu/supercomputing/software/apps/multicoil.shtml); OXHS1, OXHS2, OXHS5, OXHS7, OXHS8, and OXHS11 were predicted to be possible nuclear proteins, whereas the others were predicted to be located in cytoplasm. To check the authenticity of the prediction, we chose four representative sequences (OXHS1, OXHS2, OXHS8, and OXHS10) to make OXHS-GFP fusion constructs and transformed them into onion epithelial cells. The results showed that all the three fusion proteins were located in cytoplasm (Fig. 4), which differed from the predicted nuclear location for OXHS1, OXHS2 and OXHS8.

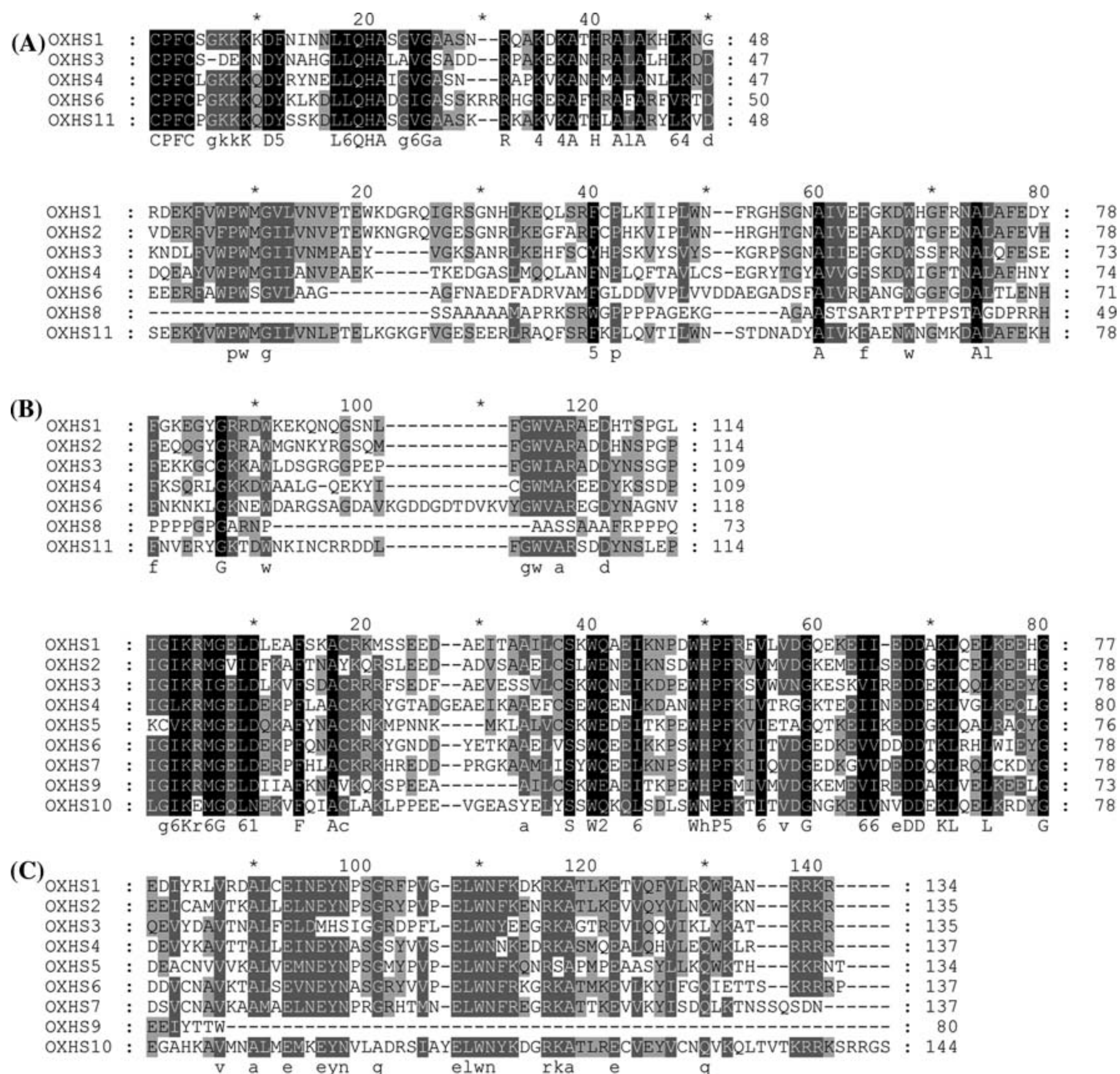

Fig. 3 Sequence alignment of the Zf-XS domain (**a**), XS domain (**b**), and XH domain (**c**) of OXHS family. The sequences are based on *japonica* rice Nipponbare. Alignment was drawn based on ClustalX software. The identical residues are indicated in *black*

Expression profiles of OXHS family in different tissues and organs

RT-PCR was performed to check the relative transcript levels of *OXHS* genes in different types of tissues or organs using gene-specific primers. Transcripts were detected for all the *OXHS* genes except *OXHS11*. No transcript was detected—not even with long PCR cycles—for *OXHS11* in all the tissues checked, and no EST was found for this gene in the rice EST database either. This result suggests that *OXHS11* may either be a pseudogene or specifically expressed in limited tissues or organs that were not included in this experiment. The other 10 *OXHS* genes can be classified into three types according to their expression patterns (Fig. 5). The first type of genes (including *OXHS1, OXHS6, OXHS8,* and *OXHS10*) were expressed in most of the tissues or organs investigated. The expression patterns of these genes have strong similarity, especially *OXHS1* and *OXHS10*, which both show high transcript levels in young panicles, stamen, pistil, root, and callus. Expression patterns of *OXHS6* and *OXHS8* are similar to *OXHS1* and *OXHS10*, respectively, although the four genes showed relatively weak expression in callus or stamen. The second type of genes (including *OXHS2*, *OXHS3*, *OXHS4,* and *OXHS9*) were expressed only in young panicles, stamen, and pistil. The third type of genes (including *OXHS5* and *OXHS7*) were expressed specifically in pistil (*OXHS7*) or stamen (*OXHS5*). *OXHS2* to *OXHS9* showed higher transcript levels in reproductive tissues or organs than in vegetative tissues. Transcript was not detected in root and

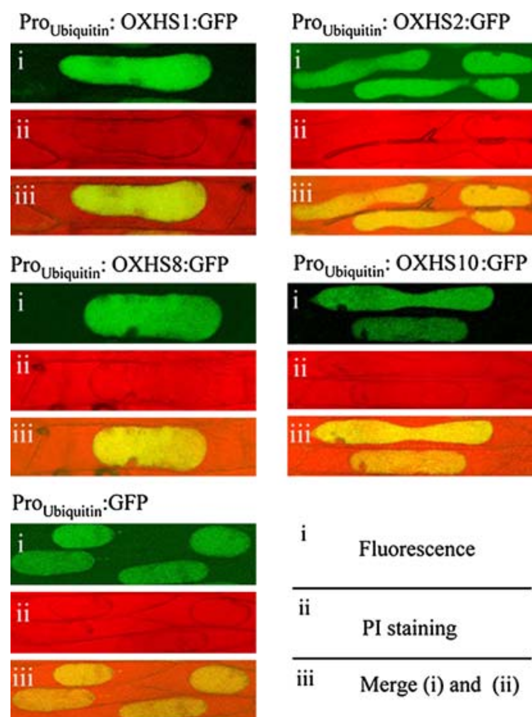

Fig. 4 Subcellular locations of OXHS proteins in onion epidermal cells. The photographs were taken under dark field for green fluorescence (*i*), bright light for the separation of cell wall and cytoplasm by 0.3% sugar solution and PI staining (*ii*). Panel (*iii*) is a merge of (*i*) and (*ii*)

Fig. 5 Semi-quantitative PCR analysis of the OXHS family in ten different tissues or organs of Minghui 63. Based on the expression levels, the *OXHS* genes can be roughly classified into three types (I–III). The rice *Actin1* gene was used as the internal control. The *numbers* after the gene names denote PCR cycles

flag leaf for *OXHS2* to *OXHS10* (Fig. 5). These results suggest that most of the genes in the OXHS family are involved in reproductive development. We also checked the expression profiles of OXHS family genes in the expression profiling database of the rice genome based on gene chip analysis for more than 30 tissues or organs (Wang et al. submitted), and the results (Supplemental Fig. 3) agreed well with the RT-PCR results.

Expression profiles of *OXHS* genes under abiotic stresses

Many plant-specific gene families are involved both in development and stress responses. To check whether any genes of the OXHS family are responsive to stresses, real-time PCR was used to quantify the relative transcript levels of the *OXHS* genes under a few abiotic stresses. The results showed that, except *OXHS5* and *OXHS11*, the other nine genes were induced in the seedling leaves by at least one of the stresses applied, including drought, salt, cold, and abscisic acid (ABA) treatment (Fig. 6). Among them, six genes (*OXHS1*, *OXHS2*, *OXHS3*, *OXHS4*, *OXHS9*, and *OXHS10*) were evidently induced by drought stress with distinct patterns. For example, the transcript levels of *OXHS2*, *OXHS3*, *OXHS4*, and *OXHS9* were increased at the early stage of drought stress and then decreased, while the expression level of *OXHS10* increased gradually and peaked at the late stage of the stress. *OXHS2* and *OXHS3* were induced by salt stress, whereas *OXHS8* and *OXHS10* were suppressed by salt stress. The expressions of *OXHS2*, *OXHS3*, *OXHS7*, and *OXHS9* were evidently induced within 3 h after cold (4°C) treatment. We noticed that the expression levels of *OXHS2* and *OXHS3* fluctuated, increasing within 3 h, being reduced to the control (0 h) level at 6 h, and then increasing more significantly at 12 h. Such a fluctuation was not due to circadian effect because these genes showed a rather constant expression level in a day-night cycle under normal growth conditions (data not shown). In the ABA treatment, *OXHS1*, *OXHS2*, *OXHS6*, *OXHS8*, and *OXHS10* were significantly induced within 3 h after treatment, and *OXHS2* showed a slight increase of transcript expression.

OXHS2 and *OXHS3* showed a similar fluctuation of transcript level in the ABA treatment as observed in the cold treatment. In fact, the stress-induced expression patterns of *OXHS2* and *OXHS3* were very similar to each other in all the four stress treatments. More interestingly, these two genes also showed similar expression profiles in different tissues or organs (Fig. 5). Further analysis of the *cis*-acting DNA elements in the promoter region (1 kb upstream of transcriptional start site) of *OXHS2* and *OXHS3* revealed a few stress-related *cis*-elements (e.g., ABRELATERD1, ACGTATERD1, ACGTTBOX) as well as a few tissue-specific *cis*-elements (e.g., GTGANTG10 and POLLEN1LELAT52 involved in pollen-specific expression). In general, the putative *cis*-elements detected in the *OXHS* genes were closely related with the expression

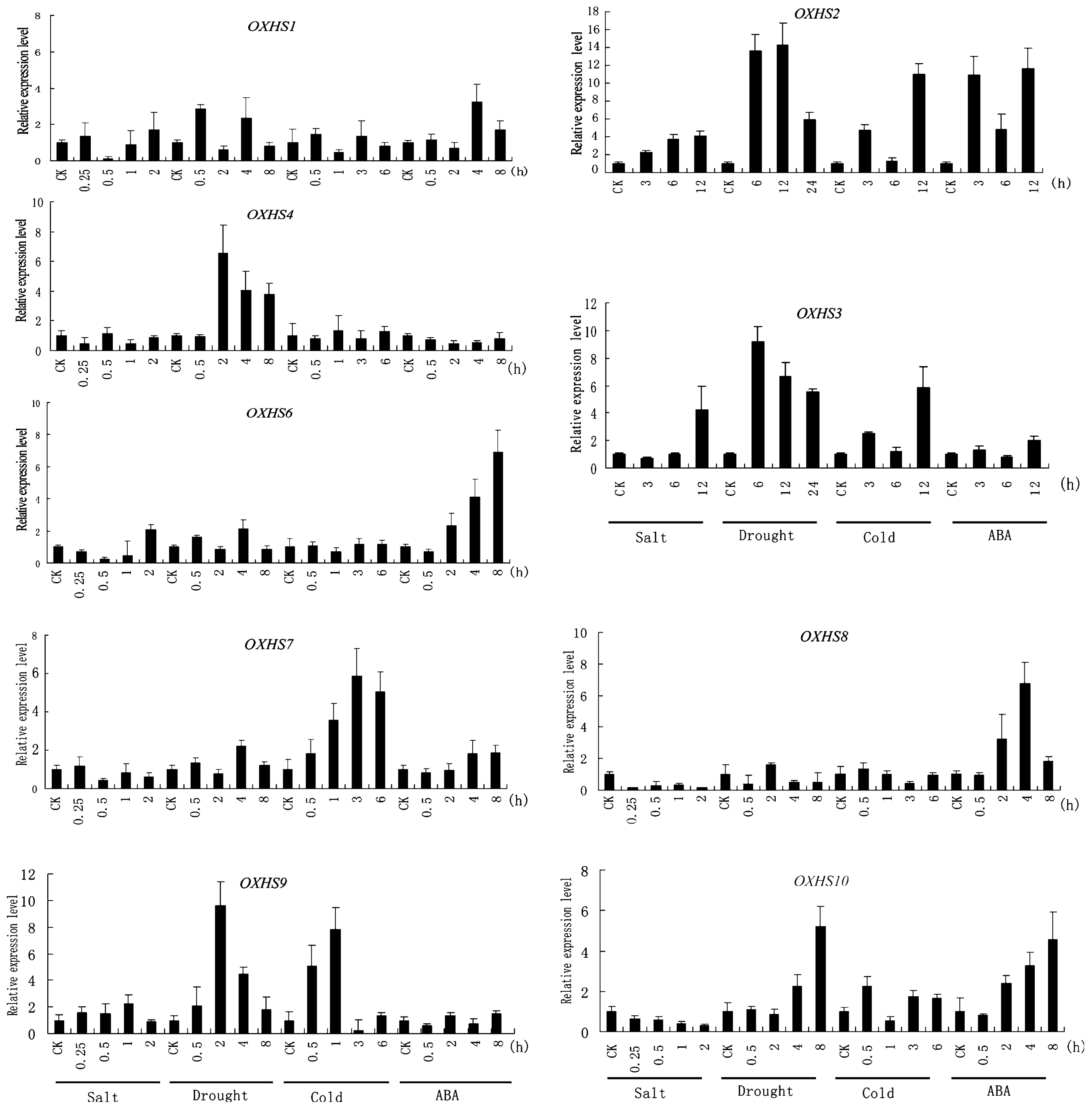

Fig. 6 Real-time PCR analysis of *OXHS* genes in response to abiotic stresses at the seedling stage in Minghui 63. Four-leaf-stage Minghui 63 was treated with drought, salt, cold, and ABA and leaves were sampled at different times as indicated. The RNA used for *OXHS2* and *OXHS3* was from a different batch of stress-treated samples. The *error bars* are based on three replications

specificity of tissues or organs and the stress responsiveness.

Over-expression of *OXHS2* in rice resulted in reduced salt and drought tolerance

As quite a few *OXHS* genes are responsive to stress treatments, we further tested whether these genes are involved in stress tolerance. For this purpose, one of the representative OXHS genes, *OXHS2*, which was strongly induced by salt and drought stresses (Fig. 6) and encodes a protein without self-activation in yeast and targeted to cytoplasm in onion epidermal cells (Fig. 4) was over-expressed in *japonica* rice Zhonghua 11 under the control of a ubiquitin promoter (Fig. 7a). Real-time PCR showed that 8 of 15 T0 transgenetic plants showed over-expression

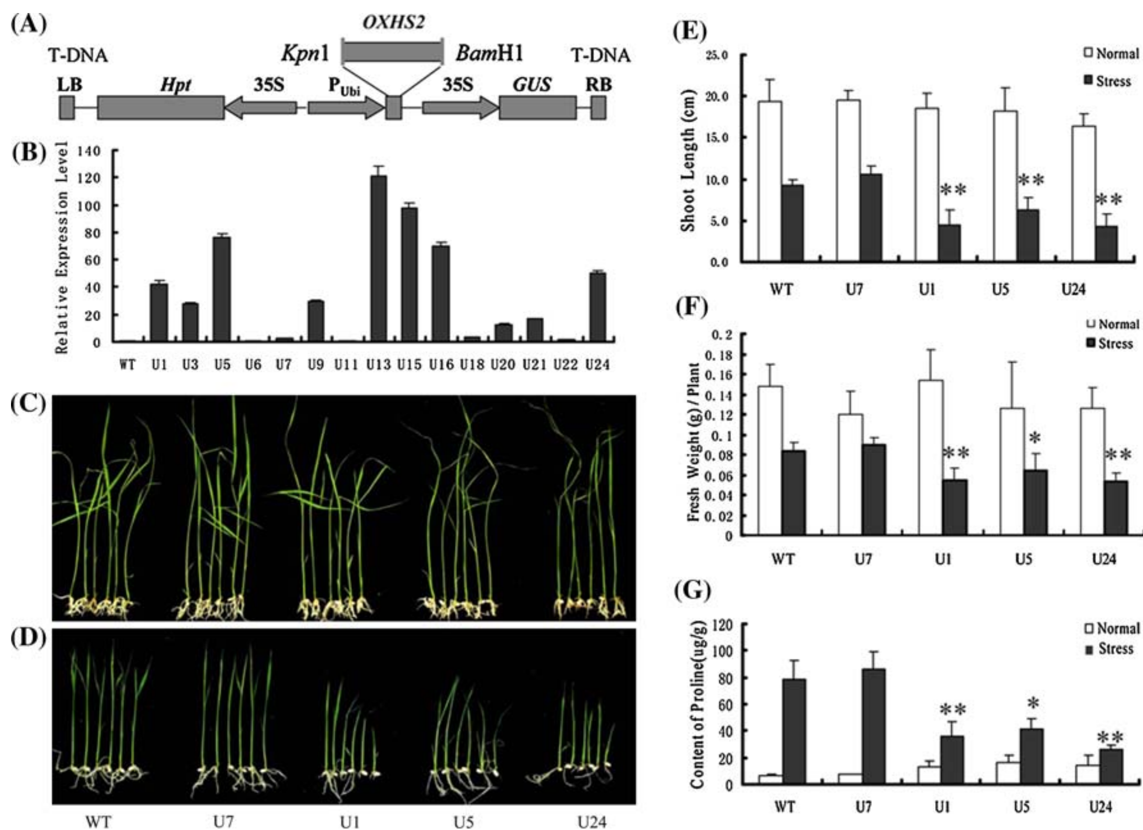

Fig. 7 Identification and salt tolerance testing of *OXHS2* over-expressed plants in rice. **a** Schematic diagram of *OXHS2* overexpression construct. Ubi, Maize ubiquitin gene promoter; Hpt, hygromycin resistance gene. **b** Real-Time PCR of part of T0 transgenic plants and WT.**c**, **d** Growth performance of WT and *OXHS2* transgenic plants under normal condition (**c**) and salt stress with 150 mM NaCl for 7 days (**d**). **e**, **f** Shoot length (E) and fresh weight (F) of WT and *OXHS2* transgenic plants under normal condition and salt stress for 7 days. **g** Content of free proline in the *OXHS2* transgenic plants compared to the WT plants under normal conditions and salt stress. U1, U5 and U24 are *OXHS2*-overexpressing lines; U7, a negative control of transgenic plant; WT, wild type. *Error bars* are based on three replicates. Significant difference was determined by *t* test (* $P < 0.05$; ** $P < 0.01$)

of the transgene (Fig. 7b). Salt stress tolerance was tested by growing three independent *OXHS2*-overexpressed T1 lines (U1, U5, and U24), one non-overexpression T_1 line (U7), and WT on MS medium containing 150 mM NaCl. On the MS medium without salt stress, the *OXHS2*-transgenic and WT plants showed no significant difference in shoot length and fresh weight (*t*-test, $P > 0.05$; Fig. 7c). Interestingly, the over-expressed lines showed obviously reduced growth performance under the salt stress (Fig. 7d). The values of shoot length and fresh weight of the *OXHS2*-overexpression lines were significantly lower than that of U7 and WT (*t*-test, $P < 0.01$, Fig. 7e, f), although the root length showed no significant difference (Fig. 7d). We measured the proline content in the leaves of the plants before and after the stress, and result showed that the stress-induced increase of proline content in the over-expression plants were significantly lower than that in U7 and WT (*t*-test, $P < 0.05$; Fig. 7g). These results suggested that the salt tolerance of the *OXHS2*-overexpressed plants has been impaired.

Drought resistance of three independent *OXHS2*-over-expression lines (U1, U5, and U24) at T_1 generation was also tested at panicle development stage (2 weeks before flowering) in the field at Hainan Island, China. The over-expression lines had significantly (*t*-test, $P < 0.05$) lower relative yield and fertility, two commonly used parameters for evaluating drought resistance at reproductive stage (Yue et al. 2006; Xiao et al. 2009), than that of the transgenic control line (U7) and WT (Fig. 8; Supplemental Fig. 4). No significant difference was observed between the over-expression lines and the controls under normal growth (data no shown). This result suggests that over-expression of *OXHS2* in rice has negative effect on drought tolerance. Nevertheless, no significant difference was detected between the *OXHS2* over-expression lines and WT for the tolerance to cold and ABA treatments, suggesting that

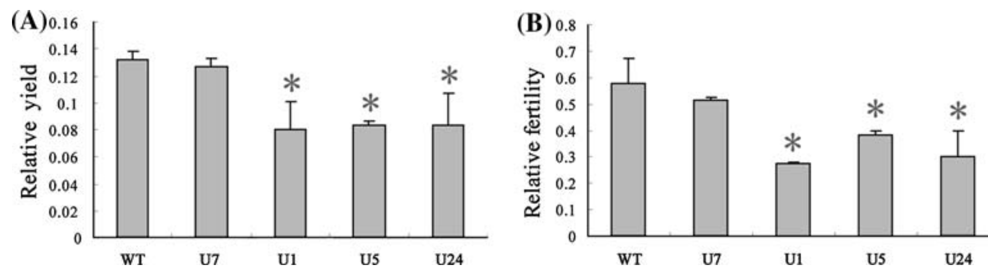

Fig. 8 Relative yield (**a**) and fertility (**b**) of over-expressed T_1 families of *OXHS2* under the field conditions after drought stress applied at panicle development stage. Relative spikelet fertility or yield is the ratio of the spikelet fertility or yield under stress conditions to that under normal growth conditions. Data represent mean ± SD ($n = 12$) with t test (* $P < 0.05$; ** $P < 0.01$)

OXHS2 gene might be specifically involved in salt and drought tolerance in rice.

Discussion

So far, no XHS homologue sequences from human, other animals, or bacteria have been deposited in the public databases, which suggests that XHS family genes may have evolved specifically in plants (Fraser et al. 2005). Using a sequence similarity search we identified 35 putative XHS protein sequences in plants, and these proteins can be divided into five subgroups (Fig. 1). Most of the XHS sequences from monocot and dicot plants are grouped separately, suggesting that XHS proteins derived from several common ancestors before the monocot-dicot divergence and the evolution of XHS proteins may be independent in monocot plants (such as rice and maize) and dicot plants (*Arabidopsis* and *M. truncatula*). Such an evolutionary feature has been also observed for other plant-specific gene families, such as SPL (Xie et al. 2006). In general, the *OXHS* genes from the same subgroup have similar gene structure. For example, *OXHS1, OXHS2, OXHS3,* and *OXHS4* in S1 and *OXHS7* and *OXHS10* in S4 have highly similar exon–intron structures, and both *OXHS7* and *OXHS10* have only the XH domain. Likewise, OXHS8 has the closest relationship with SGS3 and they both contain only the XS domain.

The XHS family is a plant-specific gene family encoding for proteins characterized by three domains: zf-XS, XS, and XH (Bateman 2002). Proteins with different domain constitutions may have distinct functions. The OXHS family can be roughly divided into three subgroups based on the domain constitutions. The zf-XS, XS, and XH domains are specific to XHS family and are highly conserved in plants. The regions beyond the three domains are variable, and no conserved motif was detected by using MEME program. These regions may contribute to the functional diversification of the proteins with the same constitution of conserved domains.

The first XHS protein (X1) was predicted to be a transcription factor (Chen and Bennetzen 1996) Analysis of the common sequence characteristics of the rice XHS proteins may provide some clues as to whether they are transcription factors. A typical plant transcription factor consists of a DNA-binding domain, an oligomerization site, a transcription regulation domain, and a NLS (Liu et al. 1999; Washburn et al. 1997). The DNA-binding domains of plant transcription factors, usually containing many basic amino acid residues, interact with DNA by binding to specific *cis*-acting elements, and such protein-DNA interactions determine the specificity or classification of transcription factors (Huang et al. 1996; Marmorstein and Fitzgerald 2003; Spittau et al. 2007; Yamasaki et al. 2008). Using motif composition analysis, we found that the C-terminal of OXHS proteins in type I (except OXHS2) and OXHS11 contain the zf-XS domain that may interact with DNA (Bateman 2002), but this possibility is challenged by the fact that some zinc finger domains mediating protein interactions are found in non-transcription factors, such as ubiquitin ligases (Imai et al. 2003; Joazeiro and Weissman 2000).

Many plant transcription factors can form hetero- and/or homo-oligomers to modulate DNA-binding specificity and affinity (Guiltinan and Miller 1994) and nuclear localization (Sainz et al. 1997a, b). Oligomers are stabilized either by hydrophobic interactions between coiled-coils and β sheets or by reactions between hydrophilic residues (Lupas and Gruber 2005; Yu 2002). The X1 protein (OXHS1) is predicted by NCoil (Lupas et al. 1991) to contain coiled-coils, which suggest that it may oligomerize (Bateman 2002). Some X1-like proteins were found to contain coiled-coil domain proteins belonging to one of five coiled-coil protein families, some of which were predicted to be involved in signal transduction in plants, while others were unknown (Rose et al. 2005). These features suggest XHS proteins may be able to form oligomers. Using the coiled-coil prediction program MultiCoil (Wolf et al. 1997), we found that nine OXHS proteins had a coiled-coil motif located between XS and XH domains, in front of the XH

domain, or behind the XS domain (Fig. 2). The coiled-coil regions contain many repeated leucine residues (Supplemental Fig. 5), which may be favorable for the stableness of the coiled-coils (Lupas et al. 1991).

Transcription factors generally have distinct actions because of the divergence in their regulation domains (Yanagisawa and Sheen 1998). Regulation domains, and hence transcription factors, function as either repressors or activators, depending on whether they inhibit or stimulate the transcription of target genes (Liu et al. 1999). Activation domains of plant transcription factors often exhibit sequence divergence; their functions depend on interactions between individual residues, and single strategically placed residues determine activation (Sainz et al. 1997a, b). For example, leucine 253 in the activation domain of the maize Myb-like transcription factor C1 is also involved in the transcription activation and modification of other amino acids in the domain (Sainz et al. 1997a, b). We tested the transactivation activity of three representative OXHS proteins in yeast and none of them showed the activity, although such activity in yeast is not a necessary requirement for all transcription factors. Nevertheless, whether the OXHS proteins have transcriptional suppression activity remains to be tested. The XS and XH domains contain a completely conserved aspartate and a glutamate site, respectively, and both sites might be enzymatically active or functionally important (Bateman 2002). Bateman suggested that XH and XS domains may interact, but our results suggested XS and XH domains could not interact in yeast.

Nuclear-localization is one of the most obvious features for transcription factors, but our results suggested that the four representative OXHS-GFP fusion proteins tested were located in the cytoplasm of onion epidermal cells. However, it cannot be excluded that the native OXHS proteins might function as co-factors in regulation are exported to nucleus by interacting with a nuclear targeting partner, as reported for a bHLH protein (Goldfarb and Lewandowska 1994).

The transcript levels of *OXHS* genes in different tissues and organs are quite diverse. All the genes are expressed in floral organs and a few of them are specifically expressed in floral organs (Fig. 5). These spatio-temporal expression profiles provided useful clues to further dissect the function of these genes in development. Noticeably, majority (9/11) of the *OXHS* genes are responsive to at least one of the abiotic stresses applied. To further confirm that the OXHS family is involved in stress tolerance, the *OXHS2* gene was over-expressed in rice, and the transgenic rice indeed showed reduced salt tolerance at the and drought tolerance. Even though the molecular functions of OXHS proteins remain unclear, which is mainly due to the limited reports on XHS family and unavailability of mutants for most of the *OXHS* genes, the facts that most *OXHS* genes are responsive to abiotic stresses and over-expression of *OXHS2* resulted in alteration of stress tolerance suggest that some members of this family may play important roles in stress tolerance. Meanwhile, some of these genes may be also involved in development control of rice as quite a few genes showed tissue- or organ-specific expression.

In conclusion, our data suggest that XH-XS-domain-containing proteins constitute a novel plant-specific family with diverse functions both in development and/or stress resistance. These results provide a general view of the organization and functions of this gene family in rice, and further elucidation of the detail molecular mechanisms of individual *OXHS* genes will greatly expand our understanding of plant development and stress responses.

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