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Comprehensive sequence and expression profile analysis of *PEX11* gene family in rice

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Abstract

PEX11 gene family has been shown to be involved in peroxisome biogenesis but very little is known about this gene family in rice. Here we show that five putative *PEX11* genes (*OsPEX11-1–5*) present in rice genome and each contain three conserved motifs. The PEX11 sequences from rice and other species can be classified into three major groups. Among the five rice *PEX11* genes, *OsPEX11-2* and *-3* are most likely duplicated. Expression profile and RT-PCR analysis suggested that the members of PEX11 family in rice had differential expression patterns: *OsPEX11-1* and *OsPEX11-4* had higher expression levels in leaf tissues than in the other tissues, *OsPEX11-2* was detected only in germinated seeds, *OsPEX11-3* was expressed predominantly in endosperm and germinated seeds, and *OsPEX11-5* was expressed in all the tissues investigated. We also observed that the rice *PEX11* genes had differential expression patterns under different abiotic stresses. *OsPEX11-1* and *OsPEX11-4* were induced by abscisic acid (ABA), hydrogen peroxide (H_2O_2), salt and low nitrogen stress conditions. *OsPEX11-3* was responsive to ABA and H_2O_2 treatments, and *OsPEX11-5* was responsive to ABA, H_2O_2, and salt treatments. However, *OsPEX11-2* had no response to any of the stresses. Our results suggest that the rice *PEX11* genes have diversification not only in sequences but also in expression patterns under normal and various stress conditions.

Keywords: *Oryza sativa*; PEX11; Expression profile; Peroxisome biogenesis; Stress

1. Introduction

Appreciation of the peroxisome as a highly versatile and important player in the cellular landscape has advanced since the "spherical and oval microbodies" were first described in the 1950s (Rhodin, 1954). Later, H_2O_2-producing peroxidases were the first enzymes found in mammalian peroxisomes (de Duve and Baudhuin, 1966) hence, they are named as peroxisomes after the H_2O_2-based respiration reaction, which is one of the conserved functions of this organelle.

In plants, peroxisomes are generally spherical but occasionally they are enlarged and clustered at the onset of seed germination and tend to elongate in dark-grown hypocotyls (Mano et al., 2002). One of the remarkable features of peroxisomes in higher plants is the plasticity of their functions. A large number of structurally similar but functionally diverse peroxisomes are involved in lipid mobilization through β-oxidation and the glyoxylate cycle, photorespiration, nitrogen metabolism, synthesis and metabolism of plant hormones, photomorphogenesis, and plant–pathogen interaction (Beevers, 2002; Lipka and Panstruga, 2005; McCartney et al., 2005; del Rio et al., 2006; Hu, 2007). Knowledge of targeting the proteins to plant peroxisomes has accumulated recently. Two types of targeting

Abbreviations: ABA, abscisic acid; H_2O_2, hydrogen peroxide; PEX, peroxin protein; LN, low nitrogen; LP, low phosphorous; PTS, peroxisomal targeting protein; PMP, peroxisome membrane protein.

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signals have been elucidated by molecular biological studies of peroxisomal matrix enzymes: PTS1 and PTS2 (Mullen, 2002). Although a few PTS2-containing peroxisomal proteins are also found in yeast and mammals, plant peroxisomal proteins usually possess significant amounts of PTS2. Extensive research in yeast (*Saccharomyces cerevisiae*) has identified a group of peroxisomal proteins called peroxins (PEXs), which mediate various aspects of peroxisome biogenesis and maintenance, including the assembly of new membrane structures, targeting of peroxisome membrane protein (PMP), matrix protein import, and peroxisome division/proliferation. At present, more than 32 *PEX* genes have been identified in yeast, whereas 20 *PEX* genes are found in mammals and 23 in plants (Sacksteder and Gould, 2000; Purdue and Lazarow, 2001; Charlton and Lopez-Huertas, 2002; Heiland and Erdmann, 2005; Mullen and Trelease, 2006; Hu, 2007). *PEX* genes are functionally conserved in higher plants (Hayashi, 2000) and of these PEX proteins, four groups of PMPs are primarily involved in peroxisome division and proliferation, among which PEX11 (formerly Pmp27) was the first to be isolated and is the best characterized. Several independent lines of evidence suggest that PEX11 proteins are directly involved in peroxisome proliferation either by representing one of the major structural components of peroxisome membrane (Erdmann and Blobel, 1995; Passreiter et al., 1998; Voncken et al., 2003), and thereby specifically shaping the membrane, or by recruiting other proteins to the membrane (Thoms and Erdmann, 2005). Absence of PEX11 leads to the presence of one or two so-called giant peroxisomes in *S. cerevisiae* cells and caused cessation of growth of cells on oleate-containing medium because of their inability to metabolize oleate (Erdmann and Blobel, 1995; Marshall et al., 1995). Conversely, over-expression of the gene caused an increase in the number of peroxisomes per cell (Erdmann and Blobel, 1995; Marshall et al., 1995). Although this protein was discovered more than 10 years ago, how PEX11 regulates the proliferation of peroxisomes is still poorly understood. It has been hypothesized that the peroxin 11-type (PEX11P) homologs, in concert with dynamin-related proteins, uniquely regulate peroxisome division or proliferation (Thoms and Erdmann, 2005). In human beings, there are three *PEX11* isoforms, among which *HsPEX11β* is constitutively expressed and essential for embryo viability, *HsPEX11α* is strongly induced by metabolic cues, and the specific function for *HsPEX11γ* is elusive (Li and Gould, 2002). Over-expression of *HsPEX11β* had greater impact on peroxisome proliferation than that of *HsPEX11α* (Schrader et al., 1998). However, whether the PEX11 protein family members in mammals are functionally equivalent, is still unclear. Despite being one of the most abundant peroxisomal membrane proteins in diverse species (Erdmann and Blobel, 1995; Voncken et al., 2003) the biochemical function of PEX11 still remains elusive.

The lack of apparent plant homologs of most yeast and mammal proteins that function in peroxisome proliferation suggests that it is essential to study this fundamental cell biological process in plants. Rice is one of the most important crops and has become a model plant of monocot species for functional genomics and gene function studies. However, limited knowledge of the *PEX11* gene family is available in this species. In this study, five putative rice PEX11 sequences were identified and compared with PEX11 homologs from other plant species by sequence and phylogenetic analysis. We determined the expression patterns of the *PEX11* gene family in the whole life cycle of the rice plant by expression profile and reverse transcription-polymerase chain reaction (RT-PCR) analyses. An attempt was also made to reveal the differential responses of the rice *PEX11* genes to various abiotic stresses by RT-PCR analysis.

2. Materials and methods

2.1. Identification of PEX11 homologous in rice

To find the *PEX11* genes in rice (*OsPEX11*), the keyword "PEX11" was first used as a query to search against the TIGR database (http://www.tigr.org/). The sequences obtained by keyword search were then used as queries to search against the TAIR database (http://www.arabidopsis.org/index.jsp) using the BLASTP program and the NCBI (www.ncbi.nlm.nih.gov/blast/) by BLAST to get the putative PEX11 homologs in *Arabidopsis* and other species, respectively. The PFAM database (http://www.sanger.ac.uk/Software/Pfam/) was finally used to confirm each predicted *OsPEX11* protein sequence as a PEX11 protein family member, sharing a common domain. Marker-based physical map of *japonica* rice chromosome was downloaded from the International Rice Genome Sequencing Project (http://rgp.dna.avrc.go.jp/IRGSP/). BACs or PACs containing *OsPEX11* genes were searched. Information about the position of the gene on the chromosome was collected from GBROWSE (http://gbrowse.ncpgr.cn/cgi-bin/gbrowse/japonica/). The distinctive name for each of the *OsPEX11* genes identified in the study was given according to its position on the *japonica* rice chromosomes (results were also similar in *indica* genotype, data obtained from RISE [http://rise.genomics.org.cn/]).

2.2. Protein sequence analysis of the PEX11 gene in different species

Protein sequences of putative PEX11 members collected from the TIGR, TAIR, NCBI, and PFAM were analyzed by EXPASY PROTOPARAM tool (http://www.expasy.org/tools/protparam. html). Information about the number of amino acids, molecular weight, theoretical isoelectric point (PI), amino acid composition, and instability index [instability index of >40 was considered as unstable (Guruprasad et al., 1990)] were obtained by this tool. The conserved domain of the PEX11 family protein sequence was determined by PFAM program (http://www.sanger.ac.uk/Software/Pfam/).

To confirm the conserved motifs in different species, protein sequences of all the species were analyzed in the MEME program (http://meme.sdsc.edu/meme/meme.html). CLUSTALX alignment also confirmed the three classes of motifs that were conserved in different species.

2.3. Phylogenetic analysis of PEX11 family

Protein sequences from the PEX11 family were subjected to sequence alignment using the CLUSTALX program; the

phylogenetic tree was constructed using PHYLIP software. We used a series of programs including Sequence Boot, Protdist, Maximum-likelihood, and Consense to obtain boot strap values, and finally MEGA 3.0 (Kumar et al., 2004) software was used to get the readable form of phylogenetic tree by conducting 1000 replications. DIVERGE program (Gu and Velden, 2002) was used to detect functional divergence between members of a protein family. A phylogenetic tree was generated and re-rooted using DIVERGE software (Gu, 1999).

2.4. Ka/Ks analysis to determine the similarity between OsPEX11-2 and OsPEX11-3 genes

Ka/Ks analysis was performed to obtain more information about the similarity between *OsPEX11-2* and *OsPEX11-3* gene sequences. Pairwise comparison of *OSPEX11-2*, *-3*, and *-4* was performed for calculating the ratio of replacement substitution rate (Ka) and synonymous substitution rate (Ks) using maximum likelihood algorithm implemented by PAML (Yang, 1997). Generally, Ka/Ks = 1 means that the genes are pseudogenes with neutral selection, Ka/Ks < 1 indicates the functional constraint with purifying selection of the genes, and Ka/Ks > 1 shows the accelerated evolution with positive selection. Information about the *OsPEX11* gene duplication was collected from the TIGR (http://www.tigr.org/tdb/) and was manually analyzed.

2.5. Expression profile analysis of PEX11 family in rice

Expression profile data of *PEX11* gene family for an elite hybrid rice parent, Minghui 63 (*indica*), was extracted from CREP database (http://crep.ncpgr.cn) and manually analyzed the expression pattern of *OsPEX11* genes. RT-PCR was carried out by extracting RNA by using the TriZol reagent (Invitrogen, Germany) according to the manufacturer's instructions. Eight tissues (1 = shoot at 2 tillers stage; 2 = leaf at secondary branch primordium stage; 3 = leaf at young panicle stage with panicle length 4–5 cm; 4 = flag leaf at 5 days before heading; 5 = endosperm at 7 days after pollination; 6 = endosperm at 14 days after pollination; 7 = flag leaf at 14 days after heading; 8 = germinating seed at 72 h after water imbibition) were selected from the 25 tissues that have been used for DNA chip profiling analysis (http://crep.ncpgr.cn). The first cDNA strand was generated by reverse transcription of 5 μg total RNA (50 μL reaction volume) using AMV reverse transcriptase (Takara Biotechnology, Japan) at 42 °C for 60 min.

All gene-specific primers were designed based on the cDNA sequences (Table 1). The specific primer for the rice *actin* gene (X15865) was used as an internal control. Reactions were performed with rTaq polymerase (Takara Biotechnology, Japan) on Gene AMP PCR system 9700 (Applied Biosystem, USA), with the following profile: 3 min at 94 °C for pre-denaturation, followed by 32 cycles of 1 min at 94 °C, 1 min at 58 °C, and 1 min at 72 °C, and a final 5 min extension at 72 °C. Each PCR was repeated three times. Reaction mixture without cDNA template was used as negative controls and rice *actin* gene cDNA fragment was used as positive controls for each gene investigated.

2.6. Differential expression of OsPEX11 gene family to different kinds of stresses

Different kinds of stresses including salt (200 mM/L NaCl), drought (dehydration of rice seedlings after growing for 3 weeks in normal condition), low nitrogen (1/10 N), and low phosphorus (1/20 P) were carried out for 3-week-old Minghui 63 seedlings. Chemical treatments including ABA (200 μL/L) and H_2O_2 (500 μL/L) were also applied to the seedling at the same stage. Dehydration stress was applied by stopping watering and re-watering when leaves became completely rolled. For salinity stress, roots of the seedlings were immersed in a nutrition solution containing 200 mM sodium chloride. For low nitrogen (LN) and low phosphorus (LP) stresses, roots of the normally grown seedlings were immersed in a nutrition solution with low nitrogen (1/10 of normal nitrogen) and low phosphorous (1/20 of normal phosphorous), respectively. ABA and H_2O_2 treatment were conducted by spraying the seedling leaves with 200 μL/L ABA and 500 μL/L H_2O_2, respectively. RNA extraction and RT-PCR analysis were carried out as mentioned in section 2.5.

3. Results

3.1. Identification of PEX11 homologs in rice

To identify the homologous genes of *PEX11* in rice, "*PEX11*" was used as key word to search against the rice genome annotation database in TIGR. The PFAM database was used to

Table 1
Basic information and RT-PCR primers of *OsPEX11* gene family

Name	Accession no. of full-length cDNA	LOC ID in annotation database (TIGR)	Intron no.	Protein length	Primer sequence [a]
OsPEX11-1	AK104570	LOC_Os03g02590	6	237	F:CAGACTGAAGAGGCCATCCA R:AGGTACAGAACAACAAGGCC
OsPEX11-2	ABF95492/AK287976	LOC_Os03g19000	0	254	F:GACATCCTCGTCCACATCGA R:GAATTCCAGTTCTTGTGAA
OsPEX11-3	AK287976	LOC_Os03g19010	0	242	F:CAAGTTCGTCCAGAACGTCA R:CATCCGCTTGAGCAGCAGCT
OsPEX11-4	AK073835	LOC_Os04g45210	0	222	F:GACGTTCCAGTACGTGTCCA R:CCTCCTGATCATGATGAGGT
OsPEX11-5	AK061048	LOC_Os06g03660	9	425	F:ATCTTGCTCTGCTTATTTTA R:GCTCCTTCTCTAGTTTCTTC

[a] Primers used for carrying out RT-PCR in the present study.

confirm the conserved domain for the *PEX11* gene family in rice and *PEX11* homologs in other species. Results suggest that both rice and *Arabidopsis* have five *PEX11* members. The five members of the rice *PEX11* family (*OsPEX11-1*–5) are distributed on chromosome 3 (*OsPEX11-1*, *-2*, and *-3*), 4 (*OsPEX11-4*), and 6 (*OsPEX11-5*) (Fig. 1A). *OsPEX11-2* and *OsPEX11-3* are located very close (782 bp apart) to each other and these genes are likely paralogues. Basic information about all the OsPEX11 family members and primers used for the RT-PCR in the present study were mentioned in Table 1. Comparison of the full-length cDNA sequences with the corresponding genomic DNA sequences suggested that the coding sequences of *OsPEX11-2*, *-3*, and *-4* were not interrupted by any introns but *OsPEX11-1* and *-5* were interrupted by 7 and 9 introns, respectively (Fig. 1A).

3.2. Protein sequence analysis of the PEX11 family

PFAM analysis suggested that protein sequences of the PEX11 family from all the species have a typical single PEX11 domain. The amino acid length of the PEX11 proteins ranges from 219 (*DmPEX11-1 in Drosophila*) to 425 (*OsPEX11-5 in rice)*. EXPASY analysis suggested that the PEX11 protein sequences from different species have large variations in isoelectric point (PI) values (ranging from 8.68 in *D. melanogaster* to 10.10 in *Zea mays*), molecular weight (ranging from 24.7 kDa in *D. melanogaster* to 47.2 kDa in rice; *OsPEX11-5*) (Supplementary Table 1). Among the 30 PEX11 sequences analyzed, OsPEX11-2, ZmPEX11 (*Zea mays*), SpPEX11 (*Saccharomyces pombe*), HsPEX11b (*Homo sapiens*), HsPEX11c, and TnPEX11 (*Tetraodon nigroviridis*) were predicted to be unstable proteins while the rest of them were predicted to be stable proteins.

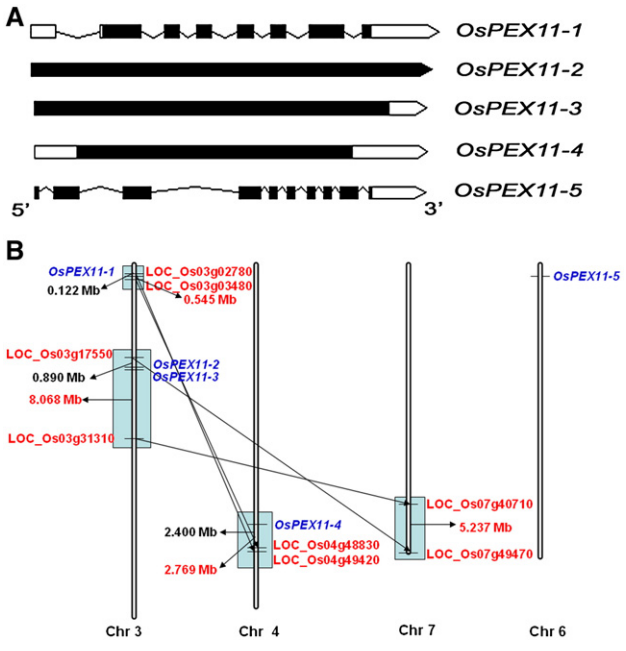

Fig. 1. Genomic distribution of *OsPEX11* genes on rice chromosomes. (A) Exon–intron structures of *OsPEX11* genes. Black box indicates exons position, white box and connecting lines indicates introns position. (B) Duplication patterns of the rice chromosomes (Wang et al., 2005) with respect to *PEX11* genes. Light green box indicates the possible duplicated segment on the chromosome. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Sequence alignment of the PEX11 family revealed three conserved amino acid regions: 200–280, 300–380, and 420–460 (Fig. 2). PEX11 sequences were further analyzed by the MEME (http://meme.sdsc.edu/meme/) program and three distinct motifs were identified (Fig. 3). Both motifs 1 and 2 have about 80 amino acids, whereas motif 3 has only 40 amino acids. The locations of the three motifs matched well with the conserved regions revealed by multiple sequence alignment analysis. Motif 1 was found in all the members of rice, three members of *Arabidopsis* (*Atpex11-1*, *AtPEX11-3*, and *AtPEX11-5*), two members of yeast (*SpPEX11* and *YlPEX11*) and one member each in other organism including human (*HsPEX11b*), medicago (*MtPEX11*), tomato (*LePEX11*), sorghum (*SbPEX11*), *Artemisia* (*AaPEX11*), maize (*ZmPEX11*), rat (*RnPEX11*), and *Drosophila* (*DmPEX11-1*). Motif 2 was found in all the sequences analyzed except *OsPEX11-3* and *AtPEX11-2*. Even though the degree of conservation of motif 3, is less than motifs 1 and 2, motif 3 was present in all the sequences analyzed except in sorghum (*SbPEX11*) (Fig. 3).

3.3. Phylogenetic analysis of the PEX11 family

30 PEX11 homologs used for phylogenetic analysis are from 19 species including seven plants species, six animal species, and six fungi species (including three yeast species). Results showed that the PEX11 sequences were classified broadly into three major groups (Fig. 4). Group I includes 15 members from fungi, yeast, and all the mammals; Group II has 10 members including all those from the plant species; and Group III has only five members with two members from *Arabidopsis* (*AtPEX11-4* and *AtPEX11-2*) and three members from rice (*OsPEX11-2*, *-3,* and *-4*). Group I was further classified into fungi and mammal subgroups and the mammal subgroup includes two clades (Fig. 4).

Functional divergence between gene clusters of the *PEX11* gene family was estimated by posterior analysis using the program DIVERGE, which evaluates shifted evolutionary rates after gene duplication or speciation. Coefficient of type II functional divergence θ between any two clusters was statistically not significant ($\theta < 0$) (data not shown).

We noticed that *OsPEX11-2* and *-3* are closely related in the phylogenetic tree. Ka/Ks analysis was performed to assess the possible recent duplication that occurred during evolution of the *PEX11* family in rice. Results indicated that *OsPEX11-2* and *-3* have 69.69% protein sequence identity and 85.25% cDNA sequence similarity; the Ka/Ks value between *OsPEX11-2* and *-3* is 0.2589 (Table 2).

As shown in Fig. 1B, a segment on chromosome 3 (0.545 Mb) containing *OsPEX11-1* was reported to have high sequence similarity with the segment containing *OsPEX11-4* on chromosome 4 (2.769 Mb), and a segment on chromosome 3 (8.068 Mb) containing *OsPEX11-2* and *-3* was found to have high sequence similarity with a segment on chromosome 7 (5.237 Mb) (Wang et al., 2005). However, no sequence similarity was found between *OsPEX11-2* and *-3* to the duplicated region on chromosome 7.

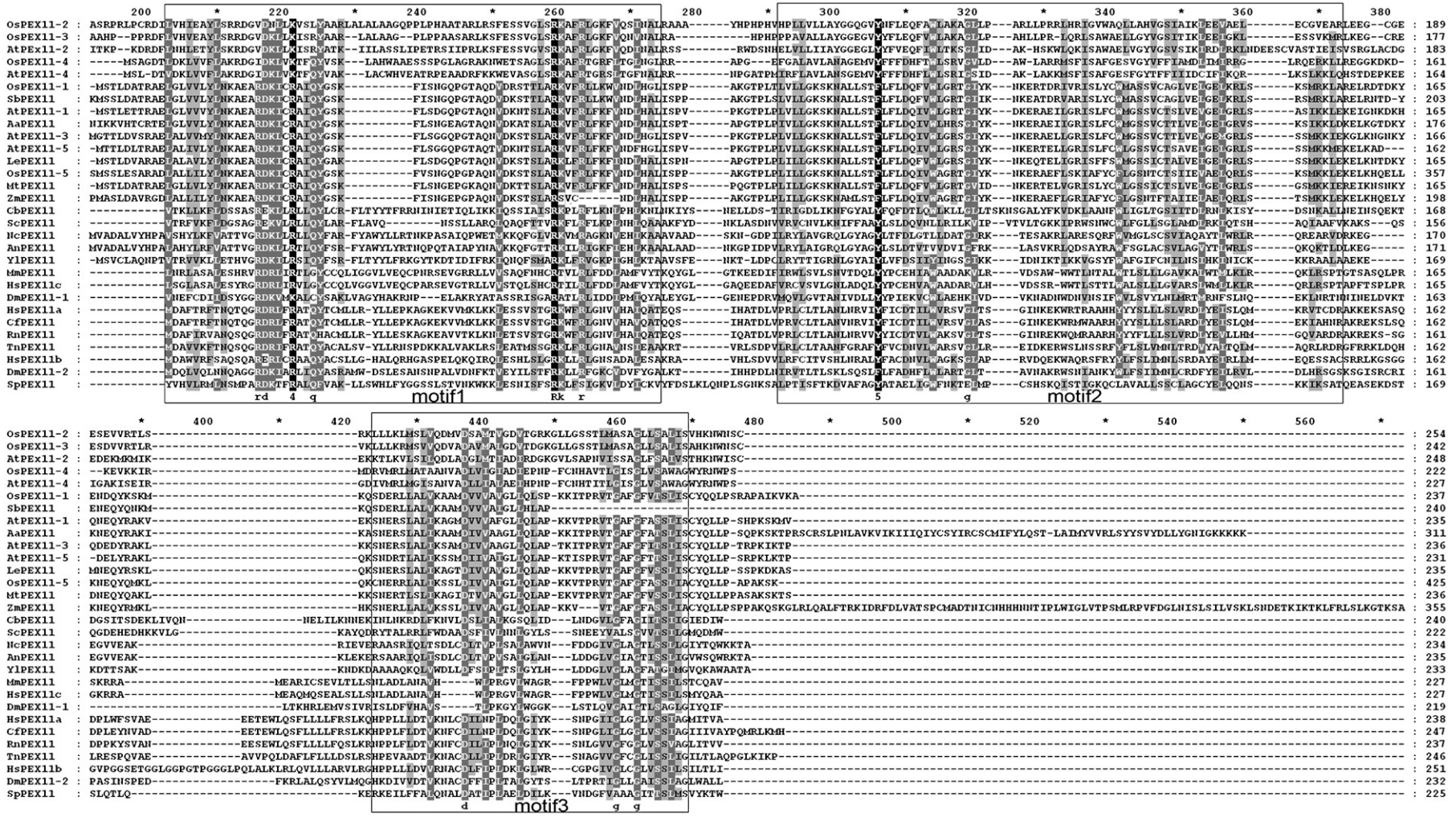

Fig. 2. Sequence alignment of rice PEX11 proteins by CLUSTALX program. Three putative motifs were indicated in boxes. The respective amino acid position is given on the top of each sequence and the species names were indicated at the left side of the figure.

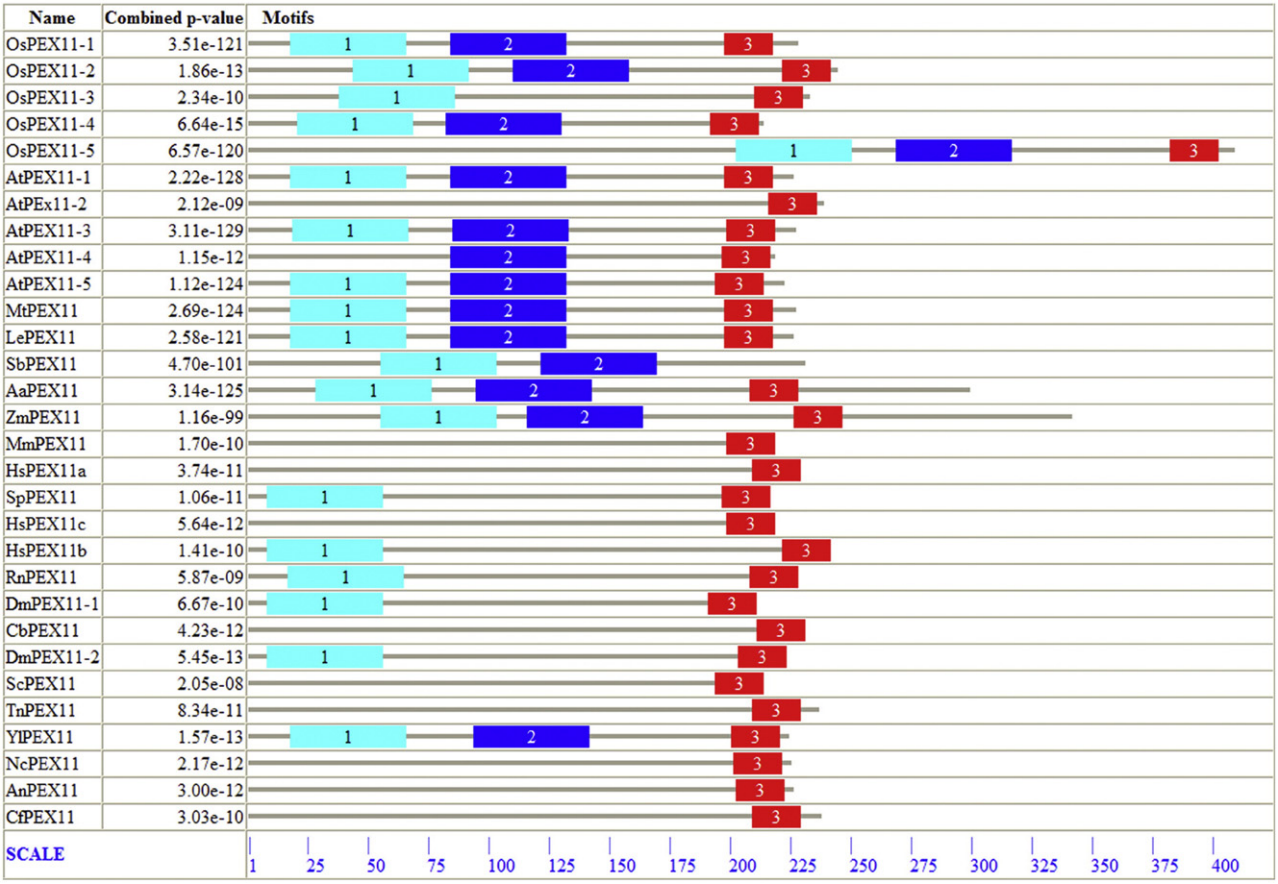

Fig. 3. Three putative motifs identified in the PEX11 family by MEME software. Motifs 1, 2, and 3 were indicated by light green, blue, and red box respectively. Name of all the members and combined *P* value were shown on the left side of the figure and motif sizes were indicated at the bottom of the figure. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Therefore, *OsPEX11-2* and *-3* may be inserted into chromosome 3 after a duplication event. No duplicated sequence was found for *OsPEX11-5* sequence in rice genome.

3.4. Expression profile analysis of PEX11 family in rice

Gene expression data of *OsPEX11* protein-encoding genes were from CREP database. The results from the database for 25 tissues with two replications indicate that the expression of *OsPEX11-1* genes was detected at the seedling stage (trefoil stage) and had highest level at the flag leaf stage (5 days before heading). The expression was also detected in vegetative tissues and the endosperm at 21 days after pollination; no expression was detected in roots and reproductive organs (Fig. 5A). The *OSPEX11-2* gene had the highest expression in the germinating seeds (72 h after water imbibition) but had showed much less or no expression in the rest of the tissues (Fig. 5B). The *OSPEX11-3* gene was expressed mainly in three types of tissues: callus and young seedling, young panicle at the secondary branch primordium and vegetative tissues at late reproductive stage (from 3 days after pollination stage to 21 days after pollination) (Fig. 5C). *OSPEX11-4* had a similar expression as that of *OSPEX11-1*, showing expression in vegetative tissues starting from the trefoil stage and also at reproductive stage until 14 days after the heading stage. The highest expression was detected in flag leaf tissue at 15 days before heading and the second highest expression was in flag leaf tissue at 14 days after heading. Faint or no expression was detected in the remaining tissues studied (Fig. 5D). *OSPEX11-5* was expressed in all the 25 tissues studied and showed highest expression in callus (resistant callus at screening stage). Apart from callus, higher expression was also detected in endosperm (at 14 and 21 days after pollination), root (at 2-tiller stage), and germinated seeds (72 h after imbibition) (Fig. 5E).

RT-PCR was performed to confirm the expression profile for some of the selected tissues that showed expression of the *OsPEX11* genes in the DNA chip microarray data. The results matched well with DNA chip data (Fig. 5F). Green fluorescent protein (GFP) reporter gene under the control of native promoter of *OsPEX11-1* gene was transformed into rice and the expression pattern of GFP in rice (mainly in leaves and immature endosperm, Supplementary Fig. 1) also supported the expression features of the gene revealed by DNA chip and RT-PCR analysis.

3.5. Differential expression of OsPEX11 gene family to different kinds of stress

To check the expression levels of *OsPEX11* family genes under abiotic stress conditions, RT-PCR was performed with total RNA isolated from 3-week-old rice seedlings subjected to

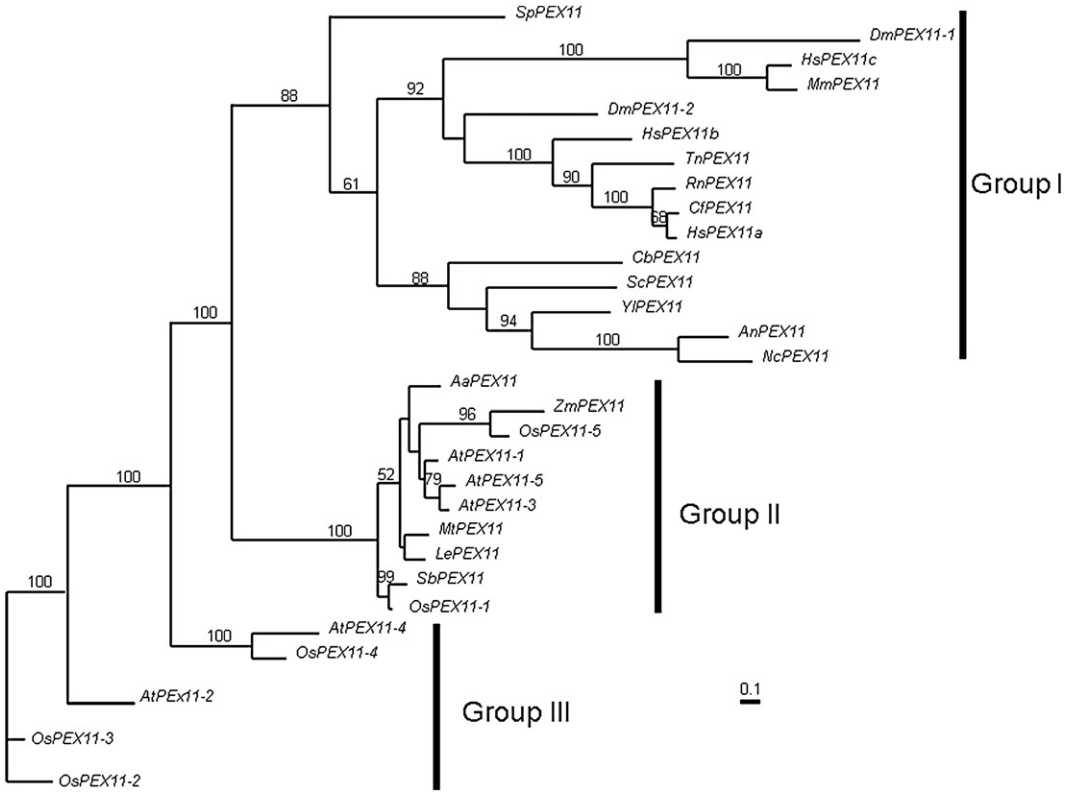

Fig. 4. Phylogenetic relationship of PEX11 members from diverse species. The unrooted tree was generated using MEGA3.0 program by Maximum-likelihood method. The bootstrap values > 50% were indicated at the branches. The abbreviations of species names were as follows: An = *Aspergillus nidulans*, Nc = *Neurospora cressa*, Yl = *Yarrowia lipolytica*, Cb = *Candida boidinii*, Sc = *Saccharomyces cerevisiae*, Sp = *Saccharomyces pombe*, Hs = *Homo sapiens*, Cf = *Canis familiaris*, Rn = *Rattus norvegicus*, Tn = *Tetraodon nigroviridis*, Dm = *Drosophila melanogaster*, Mm = *Mus musculus*, At = *Arabidopsis thaliana*, Aa = *Artemisia annua*, Le = *Lycopersicon esculentum*, Zm = *Zea mays*, Os = *Oryza sativa*, Mt = *Medicago trunculata*, Sb = *Sorghum bicolor*.

drought, salt, LN, and LP stresses, and ABA and H_2O_2 treatments at different time intervals (see "Materials and methods" for details). The results showed that *OsPEX11-2* expression was not changed in any of the stress treatments, whereas the other four genes were responsive to at least one of the stresses or treatments applied (Fig. 6).

Four *OsPEX11* genes showed expression level changes after ABA treatment. The expression level of *OsPEX11-1* was increased at 30 min after treatment, continued to increase until 12 h after treatment, and decreased thereafter. *OsPEX11-3* showed increased expression at 4 h after treatment and its expression level was gradually decreased thereafter. Both *OsPEX11-4* and *OsPEX11-5* had increased expression levels at 30 min after treatment and their expression levels began to decrease after 2 h of treatment.

In the H_2O_2 treatment, *OsPEX11-1* showed an increased expression level after 30 min of treatment and continued to increase until 12 h after treatment, and then decreased thereafter. *OsPEX11-3* had increased expression after 2 h of treatment and thereafter showed a constant increased expression level until 24 h of treatment. *OsPEX11-4* was increased at 30 min after treatment, continued to increase until 12 h after treatment, and decreased thereafter. *OsPEX11-5* showed an increased expression level at 30 min after treatment and decreased after 6 h of treatment.

In the salinity treatment, *OsPEX11-1* showed an increased expression level from 30 min to 2 h after treatment and decreased thereafter. *OsPEX11-3* showed increased expression at 30 min after treatment and decreased thereafter. *OsPEX11-4* showed increased expression at 1 h after treatment and decreased thereafter, whereas *OsPEX11-5* showed an increased expression level after 30 min of stress and continued to increase until 2 h of treatment and decreased thereafter.

For LN stress, *OsPEX11-1* showed increased expression levels at 6 and 12 h after stress and decreased thereafter. On the other hand, *OsPEX11-3* showed no expression at any time interval and *OsPEX11-4* showed an increased expression level

Table 2
Ka/Ks analysis among three members of *OsPEX11* family

SEQ1	SEQ2	Ka	Ks	Ka/Ks	PROT_PERCENTID	cDNA_PERCENTID
OsPEX11-2	*OsPEX11-3*	0.1167	0.4508	0.2589	69.69	85.26
OsPEX11-2	*OsPEX11-4*	0.6453	45.1877	0.0143	25.59	53.00
OsPEX11-3	*OsPEX11-4*	0.5691	24.7644	0.0230	35.83	55.96

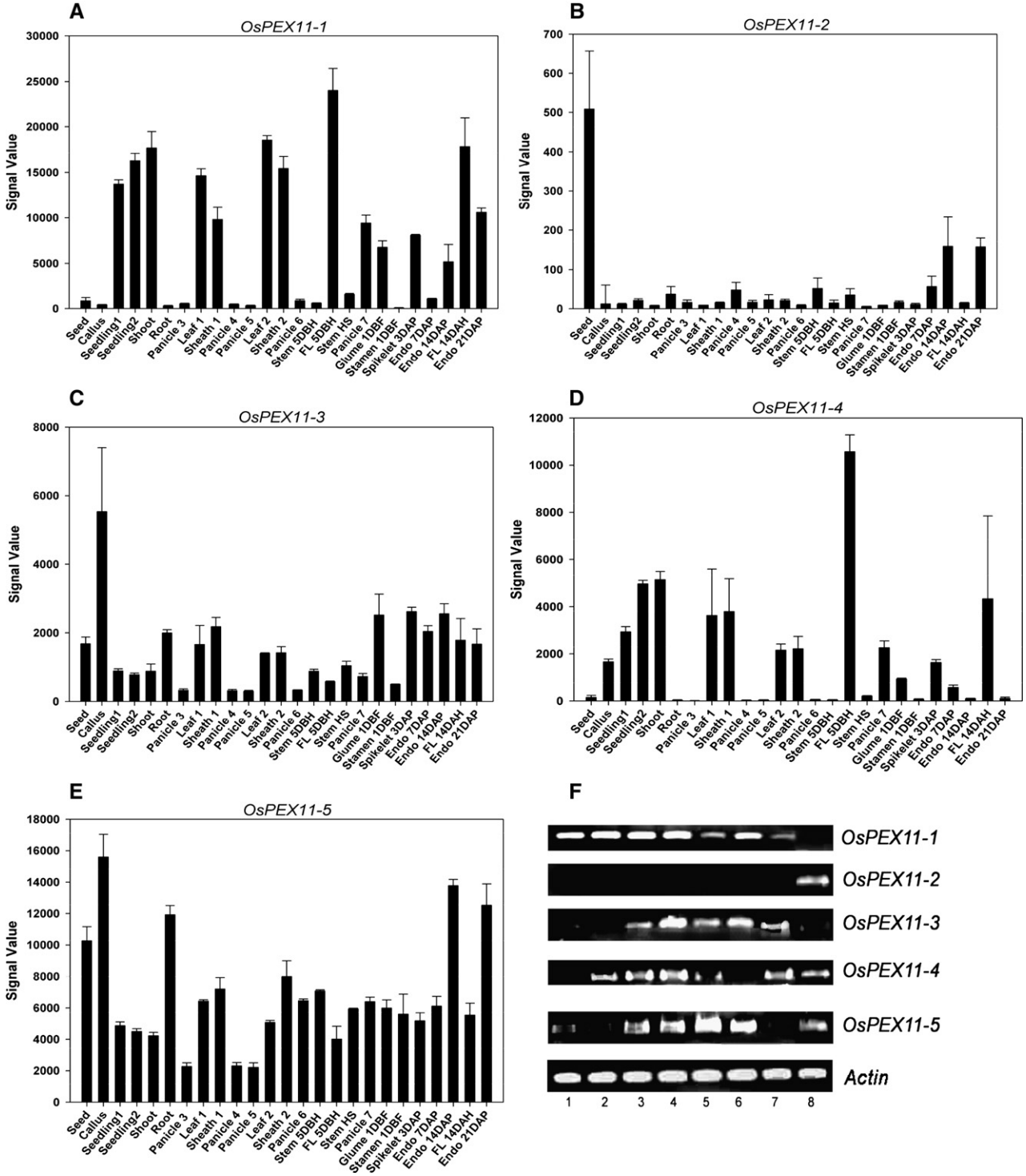

Fig. 5. Histogram diagrams of the expression patterns of the five *OsPEX11* genes in 25 different tissues (A–E) and RT-PCR analysis (F) for confirmation. Tissue names (A–E): Seed, seed at 72 h after imbibition; Callus, calli at screening stage; Seedling 1, embryo and radicle after germination; Seedling 2, leaf and root at three-leaf stage; Shoot, shoot at seedling with 2 tillers stage; Root, root at seedling with 2 tillers; Panicle 3, young panicle of secondary branch primordium differentiation stage; Leaf 1, leaf at young panicle of secondary branch primordium differentiation stage; Sheath 1, sheath at young panicle of secondary branch primordium differentiation stage; Panicle 4, young panicle at pistil/stamen primordium differentiation stage; Panicle 5, young panicle at pollen-mother cell formation stage; Leaf 2, leaf at 4–5 cm young panicle stage; Sheath 2, sheath at 4–5 cm young panicle stage; Panicle 6, panicle at 4–5 cm young panicle stage; Stem 5DBH, stem 5 days before heading stage; FL 5DBH, flag leaf at 5 days before heading; Stem HS, stem at heading stage; Panicle 7, panicle at heading stage; Glume 1DBF, hull at one day before flowering stage; Stamen 1DBF, stamen at one day before flowering stage; Spikelet 3DAP, spikelet at 3 days after pollination stage; Endo 7DAP, endosperm at 7 days after pollination stage; Endo 14DAP, endosperm at 14 days after pollination stage; Endo 21DAP, endosperm at 21 days after pollination stage; FL 14DAH, flag leaf at 14 days after heading stage. Tissue names in RT-PCR (F): 1 = shoot (seedling with 2 tillers); 2 = leaf (young panicle at secondary branch primordial stage); 3 = leaf (4–5 cm young panicle stage); 4 = flag leaf (5 days before heading); 5 = endosperm (7 days after pollination); 6 = endosperm (14 days after pollination); 7 = flag leaf (14 days after heading); 8 = germinated seed after 72 h in water.

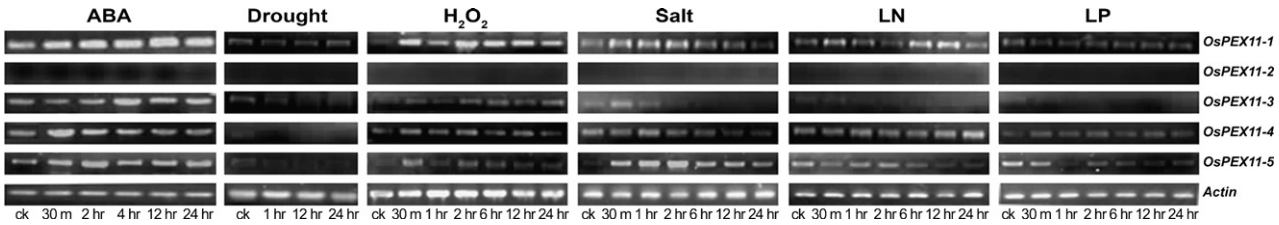

Fig. 6. Expression levels of the *OsPEX11* genes under various abiotic stresses and treatments detected by RT-PCR analysis. Time courses of stresses and treatments were indicated at the bottom of the figure, and stress type and gene name were indicated on the top and right of the figure respectively. ABA, H_2O_2 treatments and drought, salt, low nitrogen (LN) and low phosphorous (LP) stress were carried out for three-week-old seedlings. At different time intervals RNA was extracted and RT-PCR was carried out to indicate the differential expression of *OsPEX11* gene family members.

at 24 h after treatment. *OsPEX11-5* showed increased expression at 1 and 2 h after treatment and decreased thereafter.

None of the *OsPEX11* genes showed any response to the drought and LP stresses.

4. Discussion

4.1. Evolution of PEX11 gene family

Millions of years ago peroxisomes would have been primarily responsible for detoxification of cells by decreasing oxygen levels, which was then poisonous to most forms of life. Later on, peroxisomes were retained only where they performed highly specialized reactions, as they do in photorespiration, β-oxidation, and some other processes. Although peroxisomes were first found in mice (Rhodin, 1954), the fact that the majority of the peroxisomal proteomes (56% in yeast, 38% in rat) are of eukaryotic origin and the core consisting of PEX proteins contributes to peroxisome biogenesis and maintenance (Tabak et al., 2006) favors the idea that peroxisomes originated in the eukaryotic lineage during evolution. *PEX11* genes could be linked with the evolution of peroxisomes as PEX11 protein are located on the peroxisomal membrane (Li et al., 2002; Rottensteiner et al., 2003; Thoms and Erdmann, 2005; Lingard and Trelease, 2006; Orth et al., 2007).

All PEX11 sequences from 19 species containing the conserved functional domain form a group, which can be divided into fungus (including yeast), mammal, and plant subgroups, suggesting that *PEX11* genes had a single origin and later evolved independently after the divergence of these species (Orth et al., 2007). The plant PEX11 protein family appears to be the largest group (Fig. 4), and it is the largest protein family of all the PEX proteins in *Arabidopsis* and rice (Charlton and Lopez-Huertas, 2002). Recent gene duplication has occurred in both rice and *Arabidopsis* and both species have five members for *PEX11* gene family. Sequence comparison also indicates that the rice *PEX11* members *OsPEX11-2* and *-3*, possibly inserted recently into the earlier duplicated regions of chromosome 3.

The number of members for a gene family reflects a succession of genomic rearrangements and expansions due to extensive duplication and diversification that frequently occurred in the course of evolution (Wang et al., 2007). To be precise, several rounds of whole genome duplication have been found in both the *Arabidopsis* and rice genomes as reported in previous studies (Lynch and Conery, 2000; Simillion et al., 2002; Raes et al., 2003; Wang et al., 2005). Two rounds of large-scale genome duplications have been predicted in most of the rice chromosomes; one occurred around 40–50 My ago and the other occurred before the monocot–dicot divergence at about 120–150 My ago (Goff et al., 2002; Yu et al., 2002). Recently, 10 duplicated blocks accounting for 45% of rice genome sequences have been identified, indicating that rice is an ancient polyploidy (Wang et al., 2005). These results suggest that genomic duplication might be the cause for the expansion of *PEX11* gene family in rice and *Arabidopsis*.

4.2. Expression profile analysis of PEX11 family in rice

In higher plants, there are at least three defined classes of peroxisomes, whose metabolic roles are specified by the developmental state of a cell [reviewed by (Huang et al., 1983; Olsen and Harada, 1995)]. *OsPEX11-1* and *OsPEX11-4* are predominantly expressed in leaf tissues, indicating that *OsPEX11-1* and *-4* may be major *PEX11* isoforms in leaf peroxisomes that may be involved in photorespiration. Similar results have been reported in *Arabidopsis* (Tolbert, 1981; Kamada et al., 2003; Orth et al., 2007). Leaf-type peroxisomes, in green tissues, are photosynthetically active and contain enzymes essential for the light-dependent reactions of photorespiration, including glycolate oxidase and hydroxypyruvate reductase (Huang et al., 1983). As mentioned earlier, *OsPEX11-2* and *OsPEX11-3* may be recently inserted copies. Though they share high sequence similarity, their expression profiles are different (Fig. 5B–C). Such differential expression patterns imply that functional diversification may have occurred or the two genes function in different tissues or organs. It is well known that expression variation is largely due to the changes of regulatory elements. Consequently, we found absence of the TATA box in the promoter region of *OsPEX11-2*, which may be the possible reason for the unstable protein (Guruprasad et al., 1990), produced from this member. *OsPEX11-3* has high expression in seedling and senescent tissues, which is in accordance with the results of (Olsen and Harada, 1995) in *Arabidopsis*. It is well known that glyoxysomes, present in post-germination seedlings and senescent organs, function in mobilizing the storage lipids of seeds by sequestering enzymes of the glyoxylate cycle (Beevers, 1979; Huang et al., 1983; Trelease, 1984; Gut and Matile, 1988; De Bellis et al., 1990; Graham et al., 1992) thus *OsPEX11-3* can be considered as an isoform of glyoxysomes. *OsPEX11-5* shows expression in most of the tissues and can be considered as an isoform of unspecialized peroxisomes as explained for

Arabidopsis (Kamada et al., 2003). Unspecialized peroxisomes without defined metabolic roles are present in some plant organs, including the roots of most plants (Newcomb, 1982; Huang et al., 1983). Moreover, peroxisomes can undergo an interconversion of function as glyoxysomes become leaf-type peroxisomes in seedling cotyledons during the transition from heterotrophic to autotrophic growth (Titus and Becker, 1985; Nishimura et al., 1986; Sautter, 1986; Behrends et al., 1990; De Bellis et al., 1990). Thus, higher plant peroxisomes are functionally adaptable organelles that respond to the specific requirements of a cell, which is the possible reason for the *OsPEX11* members showing high expression in some specific tissues but low or no expression in other tissues.

4.3. Differential expression of the OsPEX11 genes under stresses

Plants respond to adverse environmental conditions by changing the gene expression, for eliciting various physiological, biochemical, and molecular responses. As abiotic stresses affect the cellular gene machinery, it is quite likely that the components of peroxisome membrane, such as *PEX11*, are affected as well. Even though evidence implicated peroxisomes in response to a range of biotic and abiotic stresses, a clear picture of the signaling pathways that integrate peroxisome responses to stress is lacking (Charlton et al., 2005).

ABA is a key inducer of H_2O_2 production (Hu et al., 2006) and exogenously applied ABA can cause the generation of H_2O_2 in plant cells or tissues (Guan et al., 2000; Pei et al., 2000; Jiang and Zhang, 2001; Zhang et al., 2001; Kwak et al., 2003; Hu et al., 2005). In the present study *OsPEX11* genes show increased expression after ABA and H_2O_2 treatments. Previous report suggested that rice leaves produce H_2O_2 in response to ABA (Hung and Kao, 2005), but the upstream steps of the ABA-induced H_2O_2 production pathway in rice remain largely unclear. Additionally, it has been shown that there are two sources of H_2O_2 in *Vicia faba* guard cells in response to ABA; one is the light reaction in chloroplasts, which might be the main reason for H_2O_2 production, and the other is the plasma membrane NADPH oxidase (Zhang et al., 2001). On the other hand, steady-state mRNA levels of both plant and animal peroxisome biogenesis genes increase rapidly in response to exogenous H_2O_2 application (Lopez-Huertas et al., 2000), indicating that peroxisome biogenesis is directly responsive to the major cellular stresses like H_2O_2. Therefore, treatments of diverse stresses that can generate H_2O_2 can result in peroxisome proliferation via the up-regulation of *PEX* genes required for biogenesis of the organelle and import of proteins (Lopez-Huertas et al., 2000). It is well known that all the plant peroxisomes are involved in producing and decomposing H_2O_2 (de Duve and Baudhuin, 1966; Frederick and Newcomb, 1969; Huang et al., 1983). Induction of *PEX* genes by H_2O_2 is not only confined to plants, expression of all mammalian *PEX* genes tested was significantly increased by exposure to H_2O_2, suggesting that this may be a common mechanism for dealing with oxidative stress. The higher expression of the *OsPEX11* gene members to ABA stress can also be supported by the promoter sequence analysis, which shows that many ABA-responsive elements present in all the *OsPEX11* gene members.

Peroxisomes are sensitive to external signals and are able to proliferate (Lopez-Huertas et al., 2000). Here we show that *OsPEX11-1*, *-4*, and *-5* are induced by salt and LN stresses. In addition, peroxisomes are thought to play important roles in the defense mechanism against abiotic and biotic stresses in the plants (Willekens et al., 1997; delRio et al., 1998; Barroso et al., 1999; Lopez-Huertas et al., 2000). Salt stress affects peroxisome enzyme activities (Corpas et al., 1993) and protein profiles (Lopez-Huertas et al., 1995). Peroxisomes are major sources of active oxygen species as well as the site of important antioxidant enzymes and molecules; consequently, peroxisomes are likely to play an important role in cellular redox homeostasis. Induction of peroxisome biogenesis genes by oxidative stresses fits nicely with the presence of so many antioxidant enzymes within peroxisomes and appears to be an evolutionarily ancient response of cells to the stress (Lopez-Huertas et al., 2000). Differential expression of *OsPEX11* family members under different stresses and at different stages may be related to their presence in different kinds of peroxisomes (Kamada et al., 2003; Orth et al., 2007) since previous reports suggest that *PEX11* has been implicated in peroxisomal proliferation (Erdmann and Blobel, 1995; Marshall et al., 1995; Marshall et al., 1996), which, however, needs further investigations.

4.4. Brief perspective of future study

Recent progress towards discovering the functions of the *PEX11* gene family in *Arabidopsis* sheds some light on the relation of PEX11 with peroxisome biogenesis and the present study further contributes to the better understanding of *PEX11* gene family in rice. To further reveal the functions of the *PEX11* genes in rice, we searched all the publicly available mutant databases, and mutant lines are currently available only for *OsPEX11-1* and *OsPEX11-5*. However, no obvious visible phenotypic change was observed for the mutant lines. Such a strange result was also reported in *Arabidopsis* (Orth et al., 2007). Extensive experiments are required to examine the exact biochemical roles of OsPEX11 with special attention to biological significance of the differential expression pattern of the gene family. More insight is also needed to reveal the relationship of stress responsiveness of *OsPEX11* genes with the function of peroxisome.

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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.2008.01.006.

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