A rice DEAD-box RNA helicase protein, OsRH17, suppresses 16S ribosomal RNA maturation in Escherichia coli

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1. Introduction

RNA molecules tend to form stable, non-functional secondary structures, and whose proper function requires RNA chaperones. RNA helicases are prominent candidates as RNA chaperones because the energy derived from ATP hydrolysis can be used to promote the formation of optimal RNA structures via local RNA unwinding, or by mediating RNA–protein association/dissociation (Jankowsky et al., 2001). It is known that RNA–protein interactions and the secondary structure of RNA molecules are to be important for RNA metabolism. RNA helicases are involved in ribosome biogenesis, transcription, pre-mRNA splicing, mRNA export, RNA degradation, translation initiation, and organelle gene expression. Helicases include six superfamilies (SF1 to SF6). The majority of known RNA helicases belong to the SF2 superfamily, which can be subdivided into several families including DEAD, DEAH and DExH/D (Tanner and Linder, 2001). DEAD-box proteins comprise the largest and most extensively characterized family of RNA helicases. These proteins are characterized by a core of 350 to 400 amino acids containing seven to nine conserved amino acid motifs. In the DEAD-box proteins, motif II includes the sequence D-E-A-D, from which the name was derived. The Q-motif (Tanner et al., 2003) and motifs I and II (Walker motifs A and B) are involved in ATP binding and hydrolysis (Walker et al., 1982). Motif III is speculated to couple ATP hydrolysis with helicase activity by linking conformational changes of the protein with ATP hydrolysis, which ultimately leads to RNA unwinding or RNA–protein dissociation (Velankar et al., 1999). Motif VI is believed to be involved in binding the ATP phosphates, and mutations in this motif impair ATP hydrolysis in some DEAD-box proteins (Pause et al., 1993). The solved crystal structures of a substrate-bound helicase (Kim et al., 1998) indicate that the remaining motifs (1a, 1b, IV and V) are probably involved in RNA binding and in stimulation of ATP hydrolysis by RNA binding, which are consistent with biochemical studies (Shen et al., 2007). In addition to the helicase core, DEAD-box helicases...
contain N-terminal and/or C-terminal extensions, which differ among proteins and, in some cases, target the proteins to specific sites of action via protein–protein or protein–RNA interactions.

DEAD-box proteins are found in all eukaryotes and most prokaryotes (Aubourg et al., 1999; de la Cruz et al., 1999; Rocak and Linder, 2004). Five DEAD-box proteins have been identified in *E. coli*, 25 in yeast, 32 in *Caenorhabditis elegans*, 29 in *Drosophila melanogaster*, 38 in humans, and more than 58 in *Arabidopsis thaliana*. Several studies suggest that the DEAD-box proteins play important roles in plant growth and development and in responses to biotic and abiotic stresses. For example, the *Arabidopsis* DEAD-box protein LSO4 (RH38) is essential for mRNA export and is important in stress responses and development (Gong et al., 2005). Three other DEAD-box proteins in *Arabidopsis*, RH5, RH9, and RH25, are regulated by multiple abiotic stresses. These three proteins are downregulated by salt and osmotic stress, and upregulated by cold temperatures. The overexpression of RH9 and RH25 results in retarded seed germination under salt stress, and RH25 enhances freezing tolerance (Kant et al., 2007). The homolog of Atrh5 in soybean, GmRh, which is located in the nucleus, has the same function as ATRH5 in response to low temperature and high salinity stresses (Chung et al., 2009). The loss of function of ISE1 (Atrh47), a DEAD-box protein located specifically in the mitochondria, leads to defective mitochondria by disrupting the mitochondrial proton gradient and increasing ROS (Stonebloom et al., 2009). Atrh36 plays an important role in rRNA biogenesis. The knockdown of AtRh36 results in the accumulation of unprocessed 18S pre-rRNA in the whole plant, leading to a pleiotropic phenotype (Huang et al., 2010a; Liu et al., 2010). Atrh3 and Atrh22 are involved in splicing of specific group II introns including 22S pre-rRNA and 22S rRNA and ribosome biogenesis in the chloroplast (Asakura et al., 2012). Several DEAD-box proteins, such as Atrh8 (Ts. Huang et al., 2010) and Atrh20 (Kовалев et al., 2012), are essential for virus infection. AvD1 is the dogbane (*Apocynum venetum*) homolog of *Arabidopsis* RH2, and functions as a typical helicase, with ATP-independent RNA helicase activity and RNA-dependent ATPase activity. The gene is induced by salinity and low temperature and may possess an important function in stress tolerance (Liu et al., 2008). Expression of Atrh20 homolog in tomato, ethylene-responsive 68, occurs preferentially in developing fruits and is enhanced by ethylene exposure, thereby implying an association with the regulation of genes required for cellular responses to this plant hormone. DEAD-box proteins play essential roles in basic cellular processes, such as DNA replication, DNA repair, ribosome biogenesis, transcription, pre-mRNA splicing, mRNA export, RNA degradation, translation initiation, and organellar gene expression. Therefore, DEAD-box proteins may be important in regulating plant growth and development.

Rice (*Oryza sativa*) is an important staple food crop and also a model plant for cereal genomics (Bebere-Rodrigues and Scussel, 2013; Guo et al., 2013). Studying rice DEAD-box helicases is important in elucidating the physiological importance of RNA metabolism, and will ultimately benefit rice breeding. DEAD-box proteins are highly conserved among eukaryotes, but little is known about the role of DEAD-box proteins in rice. A search of predicted protein sequences in the rice database indicated that at least 62 different genes for DEAD-box proteins exist in *O. sativa*. Several studies of rice DEAD-box proteins have been published. Li et al. (2008) identified a rice DEAD-box protein, OsBIRH1 (OsRhs0), and reported that OsBIRH1 functions in modulating defense responses against pathogen infection and oxidative stress. APIS-INTERACTING PROTEIN1 (AIP1, OsRhs6) and AIP2 (OsRhs15) form dimers and may act as bridge proteins between OsAP1 and target genes. These proteins regulate programmed cell death during tapetum degeneration by interacting with CIP promoter, a rice cytistein protease gene (Li et al., 2011). OsRh36 can restore segregation distortion in the *Arabidopsis* Atrh36 mutant, indicating that OsRh36 has the same function as ATRH36, which plays an important role in rRNA biogenesis (Huang et al., 2010b). Three other DEAD-box helicases in rice, namely, OsABP (LOC_Os06g33520), OsDBH (LOC_Os04g40970), and OsDHCT (LOC_Os11g07500), are upregulated in response to multiple abiotic stress, especially salt stress, and are regulated by the microRNAs osa-MIR414, osa-MIR408, and osa-MIR164e. These three DEAD-box proteins may be involved in responses to stress in rice (Macovei and Tuteja, 2012).

In this study, we report the characterization of rice gene OsRh17, encoding a DEAD-box RNA helicase. OsRh17 is specifically expressed in callus and pollen grains and regulated by several hormones and stresses. Prokaryotic expression of OsRh17 and two fragments of this gene, namely, N-427 and C-167 (the N-terminal 427 amino acid residues and the C-terminal 167 amino acid residues) in *Escherichia coli*, led to suppression of maturation of the precursor of 16S rRNA (pre-16S rRNA) and inhibited the growth of the host. This result suggests the roles of OsRh17 in ribosomal biosynthesis in *E. coli*.

2. Results

2.1. OsRh17 is a putative DEAD-box RNA helicase

OsRh17 (Os05g0110500) is located on chromosome 5 of the rice genome and consists of 10 exons. The full-length cDNA shows that OsRh17 encodes a putative protein that consists of 591 amino acid residues with a calculated molecular weight of 67 kDa and an isoelectric point of 9.56. Searching for conserved domains revealed that amino acids 22 to 248 and 295 to 443 in the OsRh17 protein represent two typical conserved domains, DEADc (PF00270) and HELICs (helicase superfamily C-terminal domain, PF00271) (Fig. 1A), which are composed of nine conserved RH motifs. OsRh17 contained amino acid residues D-E-A-D in motif II (Fig. 1B). Thus, we hypothesized that OsRh17 is a member of the DEAD-box class of RNA helicases. Sequence comparisons revealed that this OsRh17 was highly conserved in plant evolution. The predicted OsRh17 protein shares more than 85% amino acid sequence identity with homologous proteins from several monocots such as *Zea mays* and *Sorghum bicolor* and approximately 60% identity with proteins from dicot species including *Populus trichocarpa*, *Vitis vinifera*, *Ricinus communis*, *Medicago truncatula*, *Glycine max*, and *A. thaliana*. The relative homology of OsRh17 to proteins from different species revealed their evolutionary relationships to a certain degree (Fig. 1C).

We analyzed all identified DEAD-box RNA helicases in rice. Sequence comparisons revealed that the nine motifs of DEAD-box helicase were conserved, whereas other regions were variable. The phylogenetic tree shown in Fig. 5 illustrates that OsRh17, OsRh27, OsRh51, OsRh18, and OsRh32 were clustered together, although their sequences were variable (Fig. S2). These proteins may have similar roles in rice.

2.2. OsRh17 is localized to the nucleus

A nuclear localization sequence (NLS: KKKKQKRRKPKRRK) in the C-terminal region of OsRh17 (positions 569 to 585) was identified using the PSORT program, suggesting that OsRh17 is most likely located in the nucleus. To test this hypothesis, we used a biolistic transformation system for transient assay. 35S::OsRh17-GFP or 35S::GFP was introduced into onion epidermal cells by particle bombardment. GFP fluorescence was observed using a confocal laser scanning microscope. In onion epidermal cells transformed with 35S::OsRh17-GFP, the GFP fluorescence signal was only detected in the nucleus (Fig. 2D). By contrast, the GFP fluorescence signal was present throughout the nucleus and the cytoplasm in cells transformed with 35S::GFP (Fig. 2A–C). These results indicate that the OsRh17 protein is localized to the nucleus, and the nuclear localization may aid to determine both the function and the molecular mechanisms underlying the function of OsRh17.

2.3. Expression pattern of OsRh17

Reverse transcription-polymerase chain reaction (RT-PCR) was performed to determine the expression pattern of the OsRh17 gene in rice.

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**References**

Aubourg et al., 1999; de la Cruz et al., 1999; Rocak and Linder, 2004.

Chung et al., 2009.

Huang et al., 2010a; Liu et al., 2010; Kovalev et al., 2012.

Liu et al., 2008; Stonebloom et al., 2009.

Kant et al., 2007.

Liu et al., 2008; Stonebloom et al., 2009.

Huang et al., 2010a; Liu et al., 2010; Kovalev et al., 2012.

Li et al., 2008; Stonebloom et al., 2009.

Huang et al., 2010b.

Li et al., 2011.

Huang et al., 2010b.

Gong et al., 2005.

Gong et al., 2005.
Total RNA was isolated from a variety of tissues, including callus, seedling, root, and flower. Specific primers designed in C-terminal were used to detect the presence of OsRH17 and ubiquitin (LOC_Os03g13170) mRNA. OsRH17 is mainly expressed in callus and flower. OsRH17 expression is very low in root and almost undetectable in seedling (Fig. 2G).

To further explore the tissue-specificity of OsRH17, the reporter gene β-glucuronidase (GUS) was expressed in wild-type rice line ZH11 driven by the OsRH17 promoter, which consisted of a 2236 bp DNA fragment upstream of the OsRH17 ATG start codon. More than 20 independent transgenic plants were obtained. Three transformants that carried the transgene were selected for further analysis of GUS expression. Results of GUS expression experiment were consistent with those of RT-PCR, as the GUS expression of the transgene were selected for further analysis of GUS expression.

Northern blot analysis was used to further analyze OsRH17 expression during callus cell differentiation in rice, including the induction, pre-differentiation, and re-differentiation stages. As shown in Fig. 2H, OsRH17 transcripts gradually increased with prolonged time during induction and differentiation of callus. The highest OsRH17 expression was observed 5 days after re-differentiation and then declined afterward, which implies that OsRH17 may function in rice cellular differentiation.

2.4. OsRH17 is regulated by plant hormones and abiotic stress

Several plant hormones regulate rice cell differentiation. Different stages of callus induction require different types and concentrations of hormones. For example, 2 mg/L 2,4-D is required during the inductive phase, becomes reduced to 1 mg/L in the pre-differentiation phase, and is then replaced with 2 mg/L 6-BA + 0.5 mg/L NAA + 1 mg/L KT. Published reports show that most DEAD-box proteins play important roles in responses to stresses. To investigate whether OsRH17 expression is regulated by hormones and stress, we treated rice seedlings with NAA, 2,4-D,ABA,GA,SA,NaCl,PEG or subjected to cold temperature at the three-leaf stage. Results of real-time PCR indicated that ABA and NAA treatments resulted in OsRH17 upregulation. By contrast, 2,4-D downregulated OsRH17 expression, whereas treatment with GA, SA, NaCl, and PEG caused a weak increase in gene transcription (Fig. 3A). An analysis was carried out to determine if OsRH17 expression was induced by JA because SA and JA are well-known disease resistance-related signal molecules. q-PCR results showed that the transcription levels of OsRH17 in both seedlings and roots were mildly upregulated after JA treatment (Figs. 3A and S3). Similar results were observed in the transgenic line expressing the pOsRH17::GUS construct. Fig. 3B–M shows that NAA andABA activated the OsRH17 promoter and upregulated the expression of GUS, whereas 2,4-D suppressed GUS expression. When subjected to cold temperature or treated with PEG, and NaCl, the OsRH17 promoter showed mild activation. These results suggest that reduction in 2,4-D and increase in NAA are important for OsRH17 upregulation during cell differentiation in rice, and OsRH17 may also function in responses to stresses.

2.5. OsRH17 suppresses growth in E. coli

OsRH17 and two truncated gene fragments, namely, N-427 (contains helicase core region and all conserved motifs) and C-167, were expressed in E. coli to test whether OsRH17 functions in response to abiotic stresses. Interestingly, unlike the situation with other genes expressed in prokaryotic systems, only a very small amount of two target proteins (full-length OsRH17 and the N-427 fragment) could be
detected, and no visible C-167 fragment was observed by dying with Coomassie brilliant blue (Fig. 4A). These results suggest that OsRH17 and its fragments may be toxic to E. coli cells.

The growth rate of E. coli strains expressing OsRH17 and the two fragments were analyzed to confirm the toxicity of the proteins. Same amounts of bacterial cells were inoculated onto solid LB medium containing IPTG to induce expression of the target protein genes. Fig. 4B–C shows that the growth rates of E. coli expressing the N-427 fragment were slightly lower, and close to that of the strain carrying the empty vector. The growth of cells expressing the full-length OsRH17 and C-167 proteins were drastically reduced. By contrast, all transgenic E. coli displayed the same growth rates without IPTG. The same results were observed for growth curves measured in liquid culture. Fig. 4D shows that all transformed E. coli strains reached exponential phase about 5 h to 6 h after inoculation without IPTG induction. The empty vector control strain was slightly affected by IPTG and reached the exponential phase about 7 h after inoculation, but host cell growth was strongly influenced by the expression of OsRH17 and its fragments. The N-427 strain took 12 h to reach the exponential phase, whereas the strain expressing the full-length OsRH17 and that strain expressing C-167 required 14 h and >16 h, respectively. Results from both solid culture and liquid culture experiments showed that growth of the transgenic E. coli strains was inhibited, and the extent of inhibition was in the order C-167 > OsRH17 > N-427 > control. This result suggests that OsRH17 and its fragments were toxic to E. coli and is the reason why protein expression was in the order C-167 (none observed) > OsRH17 (weakly expressed) > N-427.

2.6. E. coli growth inhibition by OsRH17 fragments is not related to NaCl concentration

Many DEAD-box proteins are known to be involved in stress responses. Because LB medium contains 170 mmol/L NaCl, the observed growth inhibition could possibly be due to increased salt sensitivity when OsRH17 and the fragments were expressed in E. coli. To address this issue, we tested reduced levels of NaCl in the LB medium, including 150, 100, 50, and 0 mmol/L. Fig. 4E–O shows that E. coli growth was still suppressed by OsRH17 and the truncated proteins at all salt concentrations. The order of inhibition remained as follows: C-167 > OsRH17 > N-427 > control. In addition, NaCl was replaced with the same KCl concentration to investigate the effect of Na+ on inhibited growth by OsRH17. No ameliorating effect on growth was observed in the medium containing KCl (Fig. 4P–W). In the control group, there was no differences were observed among all transgenic strains under all conditions without

Fig. 2. Subcellular localization and expression of OsRH17. A–F: Onion epidermal cells transformed with the 35S promoter::GFP construct (A–C), or the 35S promoter::OsRH17-GFP construct (D–F). A and D: Bright-field images. B and E: Green fluorescence images. C and F: overlays of green fluorescence and bright-field images. G: RT-PCR analysis of OsRH17 gene expression in rice. Total RNA was isolated from callus, seedlings, roots, and flowers. The rice ubiquitin gene (LOC_Os03g13170) was used as an internal control. H: Northern blot analysis of OsRH17 gene expression during three stages of rice callus differentiation (inducing stage, pre-differentiation stage, and re-differentiation stage). I–P: GUS activity in rice transgenic lines expressing the OsRH17 promoter fusion construct; induced callus (I), differentiated callus (J), differentiating point (K), young leaf (M), root (N), flower (O), and pollen (P).
induction. These results indicate that the inhibition of E. coli growth by OsRH17 and the truncated proteins was not related to NaCl, in either salt sensitivity or Na+ concentration.

2.7. OsRH17 and its truncated protein fragments affect rRNA maturation in E. coli

Proliferation of bacterial and fungal cells has been reported to be closely related to protein synthesis, and growth of many strains is inhibited because of disrupted ribosome assembly. DEAD-box RNA helicases have important functions in ribosomal biogenesis. To explore the mechanism of growth suppression in E. coli by the OsRH17 fragments, we analyzed rRNA and pre-rRNA expression.

Specific oligonucleotide primers were designed to amplify total 16S rRNA (mature 16S rRNA and pre-16S rRNA), total 23S rRNA (mature 23S rRNA and pre-23S rRNA), pre-16S rRNA, and 23S pre-rRNA. Fig. 5 shows no changes in expression of total 16S rRNA, total 23S rRNA, and 23S pre-rRNA after induction of the OsRH17 fragments, but the pre-16S rRNA was up-regulated in the transgenic strains. Thus, pre-16S rRNA accumulated, and mature 16S rRNA levels declined when OsRH17 fragments were expressed in the bacterial cells. These results indicate that 16S rRNA splicing is suppressed by the OsRH17 fragments. The accumulation of pre-16S rRNA was in the order C-167 > full-length OsRH17 > N-427 (Fig. 5A), which suggests almost complete suppression of 16S rRNA maturation in the C-167 strain. The N-427 fragment only had a modest effect on this process. We observed that the growth rates of strains carrying the empty vector > N-427 > full-length OsRH17 > C-167, and the levels of protein expressed in N-427 > full-length OsRH17 > C-167.

2.8. Functions of OsRH17 may be redundant in eukaryotes under normal conditions

Amino acid codons are uniform in the biological world; thus, an mRNA translates to exactly the same amino acid sequence in both prokaryotes and eukaryotes. However, posttranslational modifications differ between prokaryotes and eukaryotes, and the functions of protein products may be different. It was necessary to express OsRH17 fragments in an eukaryotic cell for further exploring the functions of OsRH17.

OsRH17 fragments were inserted into two types of vectors, intracellular and secretory vectors, and transformed into the methyloptrophic yeast Pichia pastoris. When P. pastoris was cultured in medium with glycerol as carbon source, the promoters in the vectors were not induced, and similar growth was observed in all strains (Fig. 6A, C). When the promoter was induced with methanol as the carbon source, OsRH17, N-427, and C-167 were expressed, and no differences were observed between transgenic P. pastoris and the empty vector control in either intracellular expression or secretory expression (Fig. 6B, D). These results indicate that OsRH17 fragments may be nonfunctional in P. pastoris.

OsRH17 was isolated in rice, and the most accurate approach to analyze its function was to over-express and knock down the gene in rice. Full-length OsRH17, anti-sense OsRH17, and the N-427 and C-167 fragments were cloned into the BamHI sites of the binary vector pCAMBIA1390-ubi under control of the maize ubiquitin gene promoter. Several independent transgenic lines were obtained through Agrobacterium tumefaciens-mediated transformation. The transgenic lines were identified using Southern blot analysis, and transgenic expression was analyzed using Northern blot analysis (Fig. S4). Single-copy and high expression lines (low expression lines in anti-sense transgenic lines) were selected to explore the functions of OsRH17. Plant height (Fig. 7A), flowering time (Fig. 7B), panicle length (Fig. 7C), grain number of the main panicle (Fig. 7D), and thousand seed weight (Fig. 7E) were analyzed at maturity, and no visible phenotype was observed in transgenic rice. Ribosomal RNA expression was also examined in transgenic rice, Fig. 7F shows that there were no changes were observed in the rRNAs and pre-rRNAs of the transgenic rice lines. These results indicate that the functions of OsRH17 may be redundant in natural growth conditions.

DEAD-box proteins possess important roles in plant growth and development and in responses to biotic and abiotic stresses, OsRH17 was regulated by several plant hormones and stresses. To explore whether OsRH17 functions in biotic and abiotic stress tolerance, we treated transgenic lines (including OsRH17-overexpress, OsRH17-knockdown, N427-overexpress and C167-overexpress) with low temperature, salinity and drought as well as several plant hormones. Seedlings in the three-leaf stage were transferred under 10 °C for 2 weeks for cold treatment, and 42 °C for a week for heat treatment, and 20% PEG for a week for drought treatment. Seeds were germinated in 200 mmol NaCl for 4 weeks for salinity treatment, and 0.5 mg/L NAA, 100 μmol ABA and 1 mg/L 6-BA for hormone treatment. No significant difference was observed between transgenic lines and WT (Fig. S5). These results suggest that OsRH17 may be not essential for biotic and abiotic stress tolerance in germination and seedling stages.
3. Discussion

In this study, we identified the OsRH17 gene from rice, and explored its functions in both prokaryotic and eukaryotic cells. OsRH17 was homologous to the yeast DEAD-box protein Dbp7p, which is required for ribosomal biogenesis. DEAD-box proteins function in ribosomal biogenesis with a highly conserved mechanism (Linder, 2006): (1) three of the mature rRNA species are transcribed as a large pre-rRNA, and DEAD-box proteins are involved in splicing of the two internal transcribed spacers, namely, ITS1 and ITS2, and the two external transcribed spacers, namely, 5′ETS and 3′ETS; and (2) 18S and 26S rRNAs are modified by pseudouridylation and methylation guided by snoRNAs complementary to the rRNA. DEAD-box proteins reorganize the pre-ribosomal complexes by dissociating snoRNAs from the rRNAs, thereby allowing new and mutually exclusive RNA–RNA or RNA–protein interactions to occur (Kos and Tollervey, 2005). In the present study, we showed that OsRH17, as well as its truncated proteins N–427 and C–167, suppressed the growth of E. coli. Detailed analysis of RNA expression patterns indicated that OsRH17 could be involved in the ribosome synthesis. However, no significant phenotypes were observed in transgenic yeast and rice, even when subjected in some stresses. No changes were observed in the rRNAs and pre-rRNAs of the transgenic rice lines under natural condition. The results of our studies showed that OsRH17 was localized to the nucleus and specifically expressed in pollen and differentiated callus. The obtained results suggest that OsRH17 has potential functions in some specific tissues (pollen development and differentiation) in response to particular stresses, such as cold, heat, and disease.

Results of this study showed that growth of the transgenic E. coli expressing OsRH17 fragments was severely inhibited; maturation of 16S rRNA was suppressed and pre-16S rRNA accumulated, leading to ribosomes that do not assemble properly in E. coli. The E. coli genome contains five DEAD-box genes (csdA, dbpA, rhlB, rhlE and smrB). Current evidence shows that these DEAD-box proteins have crucial functions in RNA metabolism in E. coli, including mRNA processing and decay (Py et al., 1996), ribosomal biogenesis (Nishi et al., 1988), and translation initiation (Lu et al., 1999). The smrB and csdA genes are considered to be involved in the assembly of the large ribosomal subunit at low temperature (Nishi et al., 1988). The deletion of smrB or csdA results in an acute decline of free 50S subunits and accumulation of 40S particles. In addition to 50S ribosomal biogenesis, CsdA has also been proposed to play a role in the biogenesis of the small ribosomal subunit (Moll et al., 2002). DbpA, another E. coli DEAD-box protein, is likely to be involved in the biogenesis of the large ribosomal subunit, because its enzymatic activity is specifically stimulated by hairpin 92 of the 23S rRNA which lies at the peptidyl transferase center of the ribosome (Tsu et al., 2001). Interestingly, rhlB, rhlE, and dbpA can be individually deleted without a noticeable effect on temperatures only. One DEAD-box helicase can possibly substitute in the absence of another. Some DEAD-box gene deletions were combined to test this possibility. Six combinations of DEAD-box gene deletions (ΔcisdΔsrmb, ΔcisdΔrhlE, ΔcsdAΔadbpa, ΔrhlEΔadbpa, ΔrhlEΔsrmb and ΔsrmbΔadbpa) were tested, but the growth of the double mutants was similar to the single mutants. Overexpression of RhlE can correct the cold-sensitive growth of the ΔcisdA strain (Iost and Dreyfus, 2006). This data suggest that the five E. coli DEAD-box proteins have overlapping functions and one DEAD-box protein could be partially or completely replaced by the others.

Overexpression of the R331A active site mutant of DbpA in E. coli resulted in dominant slow growth and cold sensitive phenotype similar to ΔcisdA. Cells overexpressing inactive DbpA showed increased levels of 50S and 30S subunits and decreased levels of 70S ribosomes. The DbpA R331A mutant dominantly negatively suppressed the functions of wild type DbpA (Sharpe Elles et al., 2009). But the deletion of dbpA has no noticeable effect on growth, whereas the deletion of smrB and csdA results in a growth defect at low temperature. Thus, the DbpA R331A mutant has been proposed to interfere with other DEAD-box proteins (smrB and csdA) besides DbpA. Prokaryotic expression of OsRH17 fragments led to a growth defect at 37 °C, which was not observed in all single and double mutants. These results indicate that OsRH17 fragments may dominantly negatively suppress most or all of the endogenous DEAD-box proteins in E. coli, and a proposed mechanism is shown in Fig. 8. OsRH17 is a typical DEAD-box-helicase that contains a similar helicase core region to the five E. coli DEAD-box proteins. OsRH17 fragments may substitute for the proteins and interact with some important substrates that block ribosome assembly in E. coli. Both N–427 and C–167 amino acid residues inhibited the growth of E. coli. The growth defect of cells expressing C–167 was more pronounced than N–427, and more pre-16S rRNAs accumulated in E. coli expressing C–167. These results reveal that both the N- and C-terminal regions of OsRH17 could compete with the five E. coli DEAD-box proteins to divert substrates and block ribosomal assembly. The proteins or rRNAs binding to C–167 may be irreplaceable or have more important functions than those of N–427. Interestingly, the full-length OsRH17 protein contains both regions and interacts both with substrates of C–167 and of N–427. A more moderate growth defect and less pre-16S rRNA accumulation were observed in E. coli cells expressing full-length OsRH17 compared with C–167. OsRH17 is suggested to have a similar structure and enzymatic activity to E. coli DEAD-box proteins. When OsRH17 interacted with both the N- and C-terminal substrates, the complex was functional and involved in ribosomal biogenesis. However, the activity and function of the complex were extremely weak, because OsRH17 is a rice protein, and the substrates in E. coli would probably not be the best fit for OsRH17. These results imply that OsRH17 has potential roles in ribosomal biogenesis in rice. No growth defect was observed in transgenic yeast and rice expressing OsRH17 or the two gene fragments. A total of 25 DEAD-box protein genes are present in Saccharomyces cerevisiae, of which 14 are required for ribosomal biogenesis based on genetic experiments (Bernstein et al., 2006; Granneman et al., 2006). In contrast to E. coli DEAD-box proteins, the DEAD-box proteins involved in ribosomal biogenesis in yeast are highly specific and cannot be replaced by one other protein even when overexpressed, except for Dbp3 and Dbp7, which are not essential for ribosomal biogenesis (Linder, 2006). Expressing OsRH17 in yeast may cause dominantly negative suppression of Dbp7, but the deletion of Dbp7 can be complemented by other proteins. Similar results were obtained in transgenic rice; developmental differences were not observed between OsRH17-overexpressing, OsRH17-antisense, N–427-overexpressing, and C–167-overexpressing plants and empty vector plants, and rRNA expression were also similar. The following two hypotheses are proposed to explain this phenomenon. First, more than 60 DEAD-box proteins are found in rice, but little is known about their functions. Most DEAD-box proteins involved in ribosomal biogenesis in yeast have counterparts in rice, indicating that DEAD-box proteins in rice could also have important functions in ribosomal biogenesis. For example, OsRH36, a rice protein homologous to Dbp8, is required for rRNA biogenesis and megagametogenesis in rice, consistent with the function of AtRH36 in Arabidopsis (Huang et al., 2010b). The functions of OsRH17 may be redundant in rice and could possibly be replaced by other DEAD-box proteins, like Dbp7 in yeast. Phylogenetic analysis of DEAD-box proteins in rice showed that OsRH17, OsRH27, OsRH51, OsRH18, and OsRH32 were clustered together (Fig. S1), and could have similar roles in rice. Second, in contrast to OsRH36, which is expressed in all organs and tissues, OsRH17 is specifically expressed in pollen grains and differentiating calli, and OsRH17 expression was found to be regulated by plant hormones and stresses. This result implies that OsRH17 may be involved in the development of male gametophytes, cellular differentiation, and response to some stresses. However, no obvious phenotype was observed, even in stresses (Fig. S5), possibly because OsRH17 is only required in specific developmental (development of male gametophytes and cellular differentiation in this study or other as-yet unidentified physiological conditions) processes under specific environmental conditions (salt, cold, heat, drought, etc.).
example, photo-thermo-sensitive male sterility in rice could convert to fertility when subjected to low temperatures during the pollen developmental stage.

In summary, OsRH17 was specially expressed in pollen grains and differentiated calli, and regulated by plant hormones and several stresses. OsRH17 is involved in rRNA biogenesis and suppresses 16S ribosomal RNA maturation in E. coli. The functions of OsRH17 were redundant in rice under natural condition, plant hormones treatment, and abiotic stresses. Future studies should be carried out to identify the proteins that interact in vivo with OsRH17 and some treatments in specific developmental stages, such as male gametophytes and cellular differentiation.

4. Materials and methods

4.1. Plant materials and bacterial strains

The wild type rice line used in this study was ZhongHua 11 (ZH11). Rice seeds were surface sterilized and germinated on 1/2 × Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) containing 0.8% agar supplemented with 3% sucrose at 26 °C with a 16 h/8 h day/night photoperiod. Subsequently, rice plants were grown in the green-house. The E. coli strain used for prokaryotic expression in this study was DE3 (BL21), and the yeast strain for eukaryotic expression was AH109. E. coli was cultured in LB medium at 37 °C, with added 0.5 mM IPTG for foreign protein expression. P. pastoris was cultured in YPD medium containing glycerol (replaced by methanol to induce protein expression) at 30 °C.

4.2. Oligonucleotide primers

All DNA primers used in this study are listed in Supplementary Table S1.

4.3. Isolation of OsRH17 cDNA and plasmids

Total RNA was prepared from rice calli using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). First-strand cDNA synthesis was performed using 5 μg of total RNA and Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI). The full-length OsRH17 cDNA (1776 bp) was amplified by RT-PCR. To further assay the functional domains in OsRH17, we divided the full-length sequence into two fragments, namely, N-427 and C-167. N-427 and C-167 were generated with specific primers. A stop codon (TAA) was added to the 3′ end of N-427 by the reverse primer, and an initiation codon (ATG) was added to the 5′ end of C-167 by the forward primer. Overexpression and knock-down vectors were constructed to investigate OsRH17 functions in rice. The amplified CDS was digested with BamHI and inserted into the BamHI site in the binary vector pHQSN, which generated pHQSN–OsRH17, pHQSN-antisense-OsRH17 (knock-down vector), pHQSN-N427, and pHQSN-C167. In these vectors, the inserted fragments were under the control of the maize ubiquitin gene.
promoter and terminated with the NOS terminator. To synthesize the pOsRH17::GUS reporter construct, we amplified the OsRH17 promoter region (2236 bp upstream of ATG) by PCR using the primer pair OsRH17p-F and OsRH17p-R. The resulting PCR product was digested with EcoRI and Ncol and ligated into pCAMBIA-1301 to generate p1301OsRH17GUS. For the OsRH17-GFP fusion construct, the primers OsRH17GF and OsRH17GR were used to amplify the OsRH17 cDNA. The fragment was digested with EcoRI and inserted into pHQSN-eGFP.

OsRH17, N-427, and C-167 were cloned into both prokaryotic and eukaryotic expression vectors. The CDS was inserted into pProEX HTb via the BamHI site and transformed into E. coli. The CDS was also inserted between the EcoRI and KpnI sites in the vectors pPICZA and pPICZα and then introduced into yeast.

4.4. Plant transformation

Plasmids were introduced into A. tumefaciens strain EHA105 by electroporation, and rice transformation was performed as previously described (Li and Li, 2003). Transformed callus was selected on N6 medium containing 50 mg/L hygromycin.

4.5. GUS staining and GFP localization

The T2 generation transgenic plants carrying the pOsRH17::GUS construct were assayed for GUS activity. Plant material was immersed in GUS staining solution (50 mM Na-phosphate buffer, pH 7.0, 1 mM EDTA, 0.1% Triton X-100, 100 μg/ml chloramphenicol, 1 mg/ml X-Gluc, 2 mM Ferri cyanide, and 2 mM Ferro cyanide) vacuum infiltrated for 15 min, and incubated overnight at 37 °C. The material was

Fig. 6. Cell growth in Pichia pastoris with eukaryotic expression of OsRH17 fragments. A and B, OsRH17, N-427 and C-167 were construct in secretory vector; C and D were in intracellular vector. A and C, Pichia pastoris was incubated with glycerol as carbon source, which was steady by methanol to induce exogenous proteins expression. The empty vector was used as a control.

Fig. 7. Functional analysis of OsRH17 in rice. A–E: Phenotypic analysis of transgenic rice (over-expressing full-length OsRH17, expressing anti-sense OsRH17, over-expressing N-427, and over-expressing C-167). Plant height (A), flowering time (B), panicle length (C), grain number of main panicle (D) and thousand seed weight (E) were measured at maturity. Two independent lines for each transformant, and 100 individual T2 generation homozygous plants for each line were tested at maturity. F: Functional analysis of OsRH17 in rice ribosomal biogenesis. Wild type (WT) and four transformants were incubated in 1/2 MS for 2 weeks. Total RNA was purified and subjected to RT-PCR using primers specific for 18S rRNA, 26S rRNA, ETS, ITS1, ITS2, and ubiquitin (internal control).
subsequently bleached with 75% ethanol, and then observed and imaged using a Nikon stereo microscope.

For OsRH17-GFP localization analyses, the onion bulb epidermis was prepared and particle bombardment was carried out as described (Scott et al., 1999) to introduce the plasmid using a PDS-1000 biolistic device (Bio-Rad) at 1100 p.s.i. The bombarded material was cultured in MS medium for 24 h, and then observed and imaged with an Olympus IX71 inverted fluorescence microscope.

4.6. Semi-quantitative RT-PCR

Total RNA was extracted from rice tissues and E. coli cells using TRIzol reagent (Invitrogen, Carlsbad, CA). All samples were treated with DNase I. First-strand cDNA synthesis was primed with an oligo-dT primer using the PrimeScript™ first strand cDNA synthesis kit (Takara Bio, Inc.). A 10-fold dilution of the first-strand cDNA was subjected to PCR with gene-specific primers. A total of 25 to 32 reaction cycles of PCR amplification was performed. First-strand cDNA synthesis was primed with random primers to detect the expression of rRNA and pre-rRNA. The first-strand cDNA was diluted 50-fold and then subjected to PCR with primers designed to detect specific processed forms of rRNA. The primer pairs 16SF and 16SR, 26SF and 26SR, 18SF and 18SR, and 26SF and 26SR were designed to detect total 16S rRNA and 26S rRNA in E. coli, and 18S rRNA and 26S rRNA in rice, respectively, including mature rRNA and pre-rRNA.

4.7. Expression of OsRH17 under various treatments

To study the expression pattern of OsRH17 during stress, we transferred the three-leaf stage seedlings to 1/2 × MS medium containing 100 μM ABA, 50 mg/L GA, 0.5 mg/L NAA, 1.0 mg/L KT, 2 mg/L 2,4-D, 1 mM SA, 100 μM JA, 150 mM NaCl, 15% PEG 6000, and 10 °C for cold stress, with sterilized distilled water cultured at 26 °C as a control, and treated for 4 h (JA for 6 h), respectively. Total RNA was extracted. The cDNAs were synthesized from 0.5 μg of total RNA using the Prime Script™ RT reagent Kit (Perfect Real time) (Takara Bio, Inc.). The results were calculated using ABI Prism 7300 SDS software Ver. 1.3 (Applied Biosystems Foster City, CA, USA), and transcript data were normalized as an internal control. Error bars indicate the standard error of the mean. All experiments were performed in triplicate.

4.8. Northern blot analysis

RNA samples (20 μg per lane) from rice tissues were denatured at 60 °C for 20 min with an equal volume of denaturing buffer (2 × MOPS, 50% formamide, 30% (v/v) formaldehyde, and 5 mM EDTA), separated on 1.5% agarose-formaldehyde gels, and transferred to Hybond N + membranes. The digoxigenin (DIG)-labeled C-167 cDNA fragment was synthesized by PCR and used as the probe for Northern hybridization. Prehybridization, washing, and chemiluminescent detection of the blots were performed according to the manufacturer’s instructions (Roche Diagnostics GmbH, Mannheim, Germany).

4.9. Prokaryotic expression, eukaryotic expression and growth analysis

The OsRH17 CDS, N-427, and C-167 were inserted into the pProEX HTb vector (Invitrogen, Carlsbad, CA, USA), and resulting constructs were then introduced into E. coli strain BL21 plus (DE3) cells. Total proteins were induced by 1 mM isopropyl-D-thiogalactoside at 37 °C for 4 h, extracted, and then separated by SDS-PAGE. These CDS were also cloned into the pPICZa and pPICZαA vectors and transformed into P. pastoris cells by electroporation. Foreign protein expression was induced using methanol. To determine the effects of foreign proteins on host cells, we cultured the strains without inducer in medium (P. pastoris was cultured with glycerol as carbon source) until logarithmic phase. The same amount of cells was inoculated to solid medium or liquid medium containing inducers (IPTG for E. coli and methanol for P. pastoris). Cells on solid medium were observed and imaged with...
an Alphalagher HP [ProteinSimple, Santa Clara, CA], and the OD60 of the E. coli cells (liquid culture) was measured every hour to construct a growth curve. All experiments were carried out with empty vectors as control.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.gene.2014.11.025.

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