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Molecular characterization of a novel isoform of rice (*Oryza sativa* L.) glycine rich-RNA binding protein and evidence for its involvement in high temperature stress response

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Abstract

A novel full-length cDNA encoding for glycine rich (GR)-RNA binding protein (RBP) (*Osgr-rbp4*) is isolated from rice heat shock cDNA library. Amino acid sequence of the deduced protein reveals existence of RNA recognition motif (RRM) comprising of highly conserved RNA binding RNPI and RNPII domains at the N-terminus. C-terminus of this protein is rich in arginine and glycine residues. Blast search analysis on rice genome sequence database shows that GR-RBP protein family is constituted of multiple members with high level of amino acid conservation in RNA recognition motif and glycine domain regions. Similar analysis across wider biological systems from NCBI database indicated that rice GR-RBP4 has homologs in different living genera. *Osgr-rbp4* transcript in rice seedlings is constitutively expressed as well as regulated by different abiotic stresses including high temperature stress. Ectopic over-expression of *Osgr-rbp4* cDNA imparts high temperature stress tolerance to wild type yeast cells. It is shown that OsGR-RBP4 in rice leaf cells and its immunologically homologous protein in tobacco BY2 protoplasts are nuclear proteins. Upon heat shock, bulk of these proteins appears to be localized in the cytoplasm. We suggest that OsGR-RBP4 probably bind and stabilize the stress-inducible transcripts under HS conditions.

Keywords: Glycine rich-RNA binding protein (GR-RBP); High temperature; Rice; Stress response; Yeast

1. Introduction

RNA metabolism is a well-conserved functional system in living cells [1,2]. RNA binding proteins (RBPs) influence processing, transport, localization, translation and stability of mRNA molecules [2]. Some of the major categories of RBPs in plants include polyA-binding proteins (PABPs), splicing factors, chloroplastic RBPs and glycine rich (GR)-RBPs (GR-RBPs). cDNA encoding GR-RBPs have been isolated from a large number of plant species [3,4]. *Arabidopsis* genome sequence shows 27 different GR-RBP genes [5]; some of the putative *Arabidopsis* GR-RBPs are considered novel and some appear to be orthologs of specific metazoan proteins. For instance, RBM3 protein in humans and CIRP protein in *Xenopus*, mice and human appear highly homologous to specific entries in *Arabidopsis*. Related proteins have also been characterized in yeast [6]. However, experimental data to suggest what role(s) different GR-RBPs perform in plant cells is by and large lacking [5].

Structurally, plant GR-RBPs are small (16–17 kDa) modular proteins. They contain RNA recognition motifs (RRMs) mostly at the N-terminus and glycine domain (GD) at C-terminus. In most cases, alignment of amino acids in GR-RBPs is restricted in RRM and GD domains only and it is often not possible to obtain consistent alignment because of sequence variability in the regions between the conserved domains [3]. RRM [also known as the consensus-sequence type-binding domain (CS-RBD)] is comprised of 80–90 amino acids and is found in one or more copies in various RBPs. Each RRM has highly homologous RNA binding domains RNPI and RNPII. These domains show almost 60–80% identity in different RBPs [7].

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RBPs are thought to be involved in the regulation of specific mRNA species *in vivo* [8]. While RNA binding activity of GR-RBPs is shown using mostly the *in vitro* ribopolymer binding assays [9], GR-RBP related proteins are considered to recognize specific A2REs, UUAG or AU rich regions in target mRNAs [10,11]. Pull-down assays show that UV-inducible RBPA18 protein interacts with mRNA responsive to genotoxic stress [12]. Lisitsky et al. [13] noted that RBD was independently able to bind to RNA. They however also reported that the GD has a positive role in RNA binding activity of the full-length protein, although it is not sufficient for RNA binding of its own. GD domain has been thought to be involved in protein–protein interactions [14]. Binding of GR-RBPs to protein substrates is documented in some cases. Tobacco M19 protein is shown to physically interact with a DEAD box protein [15].

Numerous studies have shown that plant GR-RBP transcripts/proteins are regulated by a host of different internal and external cues. Expression of GR-RBPs is affected by various abiotic stress stimuli such as salt, drought, low temperature and ABA [3,4,16,17]. Wheat cold shock protein, wCSP1, is reportedly a GR-RBP. In *Dunaliella salina*, Zchut et al. [18] showed that GRP-1 is up-regulated while GRP-2 is down-regulated by low temperature stress. Recent work on transcript profiling of different stress-related genes using micro- and macro-arrays shows that GR-RBPs constitute a predominant component in the response of plants to different stress conditions [19–24].

There is compelling evidence showing that RBPs are stress-regulated, nucleo-cytoplasmic shuttling proteins. Yeast Npl3p is noted to be a predominantly nuclear protein that shuttles between nucleus and cytoplasm [25]. This protein associates with polyA mRNA *in vivo* [26]. Likewise, shuttling between nucleus and cytoplasm is reported for Hrp1 protein in yeast. This movement is shown to be tightly controlled by osmotic stress. Aoki et al. [27] demonstrated that *Xenopus* CIRP2 is a nucleo-cytoplasmic shuttling protein whose movement is dependent on RGG motif in the GD region. hnRNPA1 protein in human cell lines shows nucleo-cytoplasmic shuttling in a signal-mediated, temperature-dependent manner [28]. The latter protein is devoid of any conventional bipartite nuclear localization signal [29]. These reports emphasize that characteristics of both the mRNA species and major RBPs are modulated during stress response [30].

Regulation of the translational machinery has emerged as an important component in cellular stress responses [31,32]. Specific RBPs have been found to be directly associated with translation machinery (i.e. polysomes) or other critical components like eukaryotic translation initiation factor 4G (eIF4G) for stress-regulated translation [33]. *Xenopus* CIRP protein has been found to be associated with ribosomes [8]. Thus, RBPs seem to have role in stress-dependent export of mRNA from nucleus, selective translation of stress-associated genes and increased stability of related transcripts. In plants, stress regulates selective translation of specific mRNAs and shuts off the translation of housekeeping genes [34]. However, details on whether GR-RBPs are involved in this kind of response are not well worked out in plants. Maize MA16 protein is shown to be a nucleolar protein [35]. GRP protein from *Sinapus alba* (*Sa*GRP) is localized in nucleus, and its suggested roles are in ribosome biogenesis, RNA processing and editing [36]. In *Nicotiana sylvestris*, RGP-16 and RGP-3 are found to be localized in the nucleoplasm and nucleolus, respectively [37]. However, details on the intracellular shuttling of GR-RBPs in plants are largely unknown. In this study, we show that transcript corresponding to rice *Osgr-rbp4* cDNA is high temperature-regulated, and over-expression of rice GR-RBP4 in yeast cells leads to increased high temperature tolerance phenotype. We discuss that plant stress transcripts are possibly protected during stress conditions through mediation of GR-RBPs.

2. Methods

2.1. Growth and stress conditions

General growth conditions for rice (*Oryza sativa* L.) are described previously [19,38]. Rice seeds [cultivars Pusa basmati1 (PB1) and Pusa 169 (P169); obtained from Indian Agricultural Research Institute, New Delhi 110012)] were placed on wet cotton pads placed inside beakers or in plastic trays. Six- to seven-day-old seedlings were subjected to heat stress (heat shock, HS) for various specified temperatures and time intervals by placing beakers in water baths maintained at requisite temperatures. Salt stress was imposed by different concentrations of NaCl solution. Seedlings were air-dried (AD) on aluminium foil for different durations to impose desiccation stress. For low temperature (LT) stress, seedlings were placed in cold room maintained at 4 ± 2 °C for different time intervals as indicated. For DTT treatment, seedlings were transferred to beakers containing a supporting cotton pad soaked in 100 mM DTT. Tobacco BY2 cells (Nagata) were maintained as suspension culture in MS medium at 27 °C under diffuse light conditions with constant shaking. Five-day-old suspension cultures were used for the experiments.

2.2. cDNA library construction, screening and molecular cloning

Shoots of 6–7-day-old control and heat shocked P169 seedlings were employed for the construction of cDNA expression libraries. Total RNA from frozen tissues was extracted using guanidine thiocyanate [39]. mRNA was enriched using PolyATract mRNA isolation system IV (Promega, USA). cDNA was constructed and packaged using ZAP Express™ cDNA synthesis kit (Stratagene, USA). HS cDNA library was differentially screened using labeled cDNA constructed from mRNA of control and HS seedlings. Following purification of plaques, *Osgr-rbp4* was *in vivo* excised to form plasmid and sequenced from the ends using Thermosequenase kit (USB, USA). *Osgr-rbp4* cDNA from the start to the stop codon was amplified (from pBKCMV template) by PCR with the primers 5′cggaattcatggcggcgccggatgttga3′ and 5′gtaatacgactcactatagggc3′ (T7 sequence). PCR product was digested with EcoRI/KpnI (respective sequences provided in the primers) and cloned in pBCSK(−) (Stratagene, USA).

Osgr-rbp4 coding region cloned in pBCSK(−) was digested with BamHI/KpnI and ligated into pQE30 (Qiagen, Germany) for production of full-length recombinant OsGR-RBP4 protein in *Escherichia coli* strain M15. For expressing truncated OsGR-RBP4ΔGD protein, RBP domain was PCR amplified by employing AGRBP (5′cggaattcatggcggcgccggatgttga3′, containing EcoRI at 5′ end) and KpnGRP-glycine (5′ggggtac-cggagcggcgcgactgggcct3′, containing KpnI at 5′ end) as forward and reverse primers, respectively, using pBKCMV-*Osgr-rbp4* as the template. PCR fragment was digested with EcoRI/KpnI and cloned in EcoRI/KpnI cut pBCSK(−) (Stratagene, USA). RBD was released as a BamHI/KpnI fragment and subsequently ligated to BamHI/KpnI cut pQE30. Cloning was finally confirmed by restriction digestion and sequencing by using a forward primer (5′cggataacaat-ttcacacag3′) specific to the vector pQE30.

For expression in yeast, PCR fragment as of above (after digestion with BamHI/KpnI) was cloned in BamHI/KpnI cut pGV8. For yeast transformation, this construct was linearized by EcoRV. Cloning was finally confirmed by restriction digestion and sequencing by using forward primer specific to the vector pGV8 [38]. For transformation of yeast cells, general protocols as given by Sambrook and Russell [40] were followed.

2.3. In silico analysis of rice genome for OsGR-RBP4 homologues

Nucleotide and protein sequences were analyzed using DNA analysis software DNAStar. These sequences were searched for their homology with the previously existing sequences in the NCBI database using the BlastN and BlastP programs [41]. Multiple alignments were done using MegAlign module of DNAStar. For protein domain analysis, sequences were searched against Simple Modular Architectural Research Tool (SMART) database [42,43]. Hydropathy curve of the protein was plotted using the PROTEAN module of DNAStar program. EditSeq module of DNAStar was used for translating the DNA sequence of OsGR-RBP4. The deduced amino acid sequence of OsGR-RBP4 was aligned with other GR-RBPs of rice and other organisms using CLUSTAL-V program in MegAlign module of DNAStar with residue weight set at PAM250 and gap length penalty set at 10.

2.4. Southern analysis

Southern analysis was carried out using genomic DNA isolated from rice seedlings as done previously [38]. Ten micrograms of genomic DNA was digested with BamHI, EcoRI, HindIII and KpnI. The digested DNA was resolved on 1% agarose gel, transferred by capillary action to a nylon membrane (Genescreen Plus, NEN, USA) according to Sambrook and Russell [40] and immobilized using a UV cross-linker (Startagene, USA). The membrane was probed with the full-length *Osgr-rbp4* probe at high stringency. PCR amplified *Osgr-rbp4* fragment was resolved on 1% tris-acetate-EDTA agarose gel and eluted from the gel using gene clean kit (BIO101 Inc., USA). The eluted fragment was radiolabeled with αP^{32} dCTP (Board of Radiation and Isotope Technology, India) using Megaprimer labeling kit (Amersham-Pharmacia, UK) or random primer extension labeling (NEN Life Science Products Inc., USA).

2.5. Northern analysis

Total RNA from 6- to 7-day-old rice seedlings was isolated as described above and 15 μg of total RNA was electrophoresed on a 1% agarose gel made in 1× morpholinopropanesulphonic acid (MOPS) and 1.2% formaldehyde. RNA was transferred to the nylon membrane by capillary action [38]. The membrane was hybridized with radiolabeled PCR amplified *Osgr-rbp4* fragment. Hybridizations were carried out at different stringency conditions as indicated.

2.6. Soluble protein isolation and Western blotting

Soluble proteins were extracted from the shoots of rice seedlings as per the protocol of Zivy et al. [44]. Soluble proteins were precipitated with 8 vol of chilled acetone containing 10 mM 2-mercaptoethanol to remove some of the non-proteinaceous moieties. The precipitated proteins were dissolved in Laemmli buffer. Proteins from the embryos were extracted by direct homogenization in Laemmli buffer. Protein quantification, gel electrophoresis (7.5% uniform acrylamide concentration) and Western blotting were performed. Following protein quantification [45], soluble proteins were resolved on a 15% SDS-gel for western analysis. The protein was electro-transferred on a Hybond C-super membrane (Amersham-Pharmacia) according to the protocol by Towbin et al. [46]. Mouse antiserum was raised against His6-tagged OsGR-RBP4ΔGD protein and affinity purified against Ni-NTA agarose (Novagen, USA). Cross-reaction of primary and secondary antibodies with the blot was carried out as mentioned earlier [38].

2.7. Yeast thermotolerance assays

Different yeast strains [namely FY3 (wild type), FY3-pGV8 and FY3-OsgGR-RBP4 (FL)] grown in rich YPD medium (overnight, 25 °C), were used to initiate a fresh culture in YPD. The cell density at the time of fresh inoculation was adjusted to 1×10^6 cells ml^{-1}. After two rounds of cell multiplication (nearly 4 h), cells were subjected to HS for 30 min at 50 °C. Subsequently, samples were cooled on ice for 5 min and 5 μl of 10-fold serial dilutions were spotted in YPD plates. The plates were incubated at 27 °C for 36 h to score the colonies.

2.8. Polysomal fraction analysis

For isolation of polysomal proteins from heat shocked rice seedlings, 7-day-old rice seedlings were heat stressed at 45 °C for 3 h and frozen in liquid N_2. Unstressed control samples were also harvested. Approximately 4–5 g tissue for each sample was employed for isolation of polysomal proteins. Modified protocol of Blobel and Sabatini [47] was used for the isolation

of polysomal proteins from rice. The cytosolic polysomes were collected from the bottom of the sucrose cushion. The protein pellet was dissolved in Laemmli buffer. Protein sample was precipitated by 8 vol of chilled acetone. Thirty-five micrograms of polysomal protein was used for Western blot analysis as indicated.

2.9. Immunolocalization of OsGR-RBP4

For immunolocalization, dehusked surface-sterilized PB1 seeds were inoculated on MS-basal agar medium for 10 days under culture room conditions. The aerial parts of the seedlings were excised and the unopened leaves and the stem were used for protoplast isolation. Tissues placed in Petri dish were shredded in protoplast extraction buffer (EB; 25 mM MES pH 5.5, 2 mM $CaCl_2$, 600 mM mannitol) supplemented with 0.5% macerozyme (Yakult Honsha Co. Ltd., Japan) and 1% cellulase (Yakult Honsha Co. Ltd.) [48]. BY2 cells in exponential growth phase were centrifuged at 150 × *g* (Remi Equipments, India) for 2 min. The cell pellet was re-suspended in appropriate volume of EB supplemented with 0.5% macerozyme (Yakult Honsha Co. Ltd.) and 1% cellulase (Yakult Honsha Co. Ltd.) for 2 h under culture room conditions with mild shaking (45 rpm). Rice mesophyll and BY2 protoplasts were fixed for at ~1 h in 3.7% formaldehyde solution made in 1× PBS at RT. The protoplasts were washed twice in 1× PBS and resuspended in appropriate volume of 1× PBS. The cells were washed twice with 1× PBS and then blocked for 30 min in blocking buffer (1× PBS containing 1% BSA). Primary antibody (1:200) was diluted in fresh blocking buffer and probing was done O/N at 4 °C on shaker. Secondary probing was done with Alexa Fluor488 labeled anti-mice secondary antibodies (Molecular Probes, USA) diluted 1:200 in blocking buffer. The cells were stained with DAPI (1 μg ml^{-1}) for 3 min after which the cells were washed 5–6 times to remove excess DAPI. Samples were viewed under NIKON eclipse TE300 fluorescence microscope. The wavelength characteristics used for Alexa Fluor488 was as follows: excitation 460–500 nm, dichoric mirror 505 nm and emission at 510 nm and for DAPI, UV filter (wavelength characteristics of excitation 340–380 nm, dichoric mirror 400 nm and emission at 435–485 nm) were used.

3. Results

3.1. Osgr-rbp4 cDNA isolation and in silico analysis

Rice HS cDNA library was screened differentially by labeled cDNA constructed using mRNA of control and heat-shocked rice seedlings. Following purification of plaques, rescued plasmids were randomly sequenced. In this process, number of different cDNA clones like *Oshsp17.9*, *Oshsp17.3*, *Oshsp16.9* and *Osgr-rbp* were isolated. Nucleotide sequence of *Osgr-rbp* clone (EMBL accession no. AJ302060) encodes for 194 amino acids long ORF (19.475 kDa; 7.05 p*I* point). Deduced amino acid sequence showed distinctive RRM at its N-terminus and GD at the C-terminus (Fig. 1B). RRM was composed of two RNA binding consensus sequences namely RNPI [RGFGFVTF; (K/R)G(F/Y)(G/A)FVX(F/Y)] and RNPII [CFVGGL; (C/I)(F/Y)(V/I)(G/K)(G/N)L]. GD consisted of an RGG and three GGYGG boxes (two complete; one incomplete) (Fig. 1B and C). Hydropathy curve of the protein showed that OsGR-RBP has relatively hydrophilic N-terminal and hydrophobic C-terminal ends (Fig. 1B). There are four rice cDNAs corresponding to *Osgr-rbp* deduced amino acid sequence in the GenBank [AF010579 (NCBI, direct submission), AJ002893 [49], AJ002894 [49] and NP_914833 (NCBI, direct submission)] that show notable homology (Supplementary data 1). NP_914833 was a whole genome shot gun sequence and it has since been removed and has been superceded by a new assembly of the genome in the NCBI database. On closer examination, we find that NP_914833 sequence is exactly same as AJ002894. Considering AF010579, AJ002893 and AJ002894 entries as OsGR-RBP1, OsGR-RBP2 and OsGR-RBP3 (in the chronological order with respect to NCBI submission dates), *Osgr-rbp* cDNA isolated in this study is herewith designated as OsGR-RBP4. Further, rice GR-RBP gene family appeared increasingly more complex when *in silico* analysis of the KOME cDNA database (http://cdna01.dna.affrc.go.jp/cDNA/; [50]) was carried out (Fig. 1A). There are 10 entries in KOME database (AK058443, AK059164, AK059446, AK064923, AK070016, AK072673, AK103069, AK111046, AK119238 and AK119242; there is another entry AK121668 in KOME database which we did not include in our list because sequence of this entry is exactly same as AK059446) which contain both the RBD and GR domains (there are additional entries for proteins which have either RBD or GR domains in KOME database which we have not considered in our this analysis). Characteristics of various rice GR-RBPs as noted above are summarized in Supplementary data 2. AJ002894 in NCBI database was found identical to AK059446 in KOME database. NCBI database search revealed that OsGR-RBP4 has significant similarity to TaGR-RBP (AAA75104), ZmGR-RBP (AAB88616), HvGR-RBP (AAB07749), SbGR-RBP (AAG23220), HsGR-RBP (Q14011), AtGR-RBP (NP_850017), RtGR-RBP (AAH69219) and XlGR-RBP (JC6571) (Fig. 1C). Phylogenetic analysis showed that OsGR-RBP4 was evolutionarily closest to ZmGR-RBP (Fig. 1D).

3.2. Southern analysis of Osgr-rbp4 gene in rice genomic DNA

Complexity of the *Osgr-rbp* gene family was addressed to by Southern blotting. Rice genomic DNA digested with four different restriction enzymes (namely BamHI, EcoRI, HindIII and KpnI) was probed using full-length *Osgr-rbp4* cDNA. All lanes showed practically a smear when hybridization was carried out at 42 °C in the presence of 50% formamide. Distinct bands were not observed even though hybridization temperature was raised to 50 °C. However, when the hybridization temperature was further increased to 58 °C, a clear picture of cross-reacting DNA bands emerged (Fig. 2). At this temperature, DNA blot profile indicated that a small family of genes probably encodes *Osgr-rbp*.

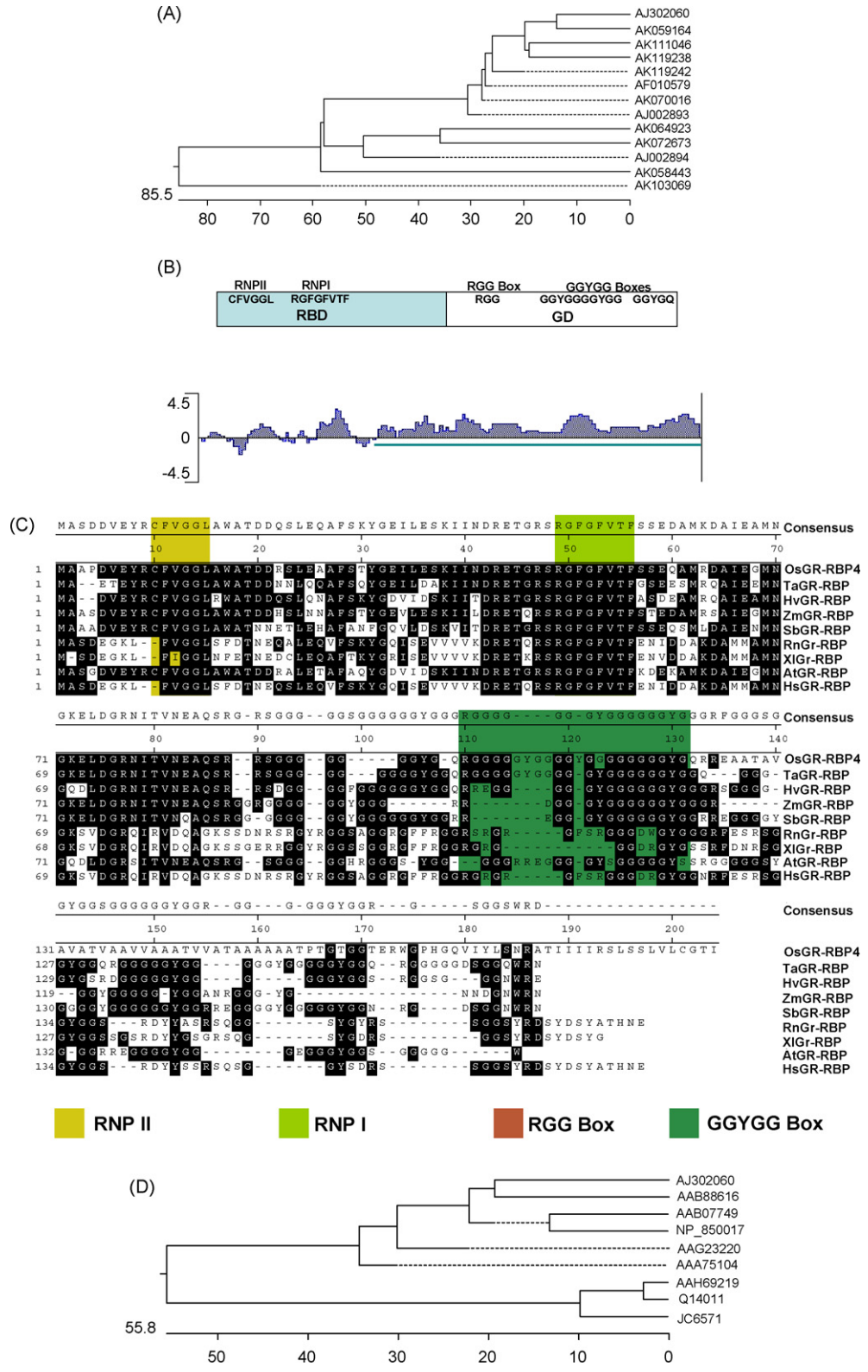

Fig. 1. (A) Phylogenetic relationship of rice GR-RBPs as determined by CLUSTAL-V of DNAStar. The length of each pair of branches represents the distance between sequence pairs, while the units at the bottom of the tree indicate the number of substitution events. AJ302060 (OsGR-RBP4) was isolated in this study. (B) Schematic model of the OsGR-RBP4 protein. RNPI and RNPII sequences present in RBD domain as well as RGG and GGYGG boxes noted in GD are shown. Hydropathy plot of OsGR-RBP4. Bold line underneath the plot depicts the region of the protein that has highly hydrophobic stretch of amino acids rich mainly in glycine residues. (C) Alignment report of homologous GR-RBPs reported from different plant and animal systems. Full-length amino acid sequence of various proteins was obtained from NCBI database. AJ302060—accession from *O. sativa*; AAB88616—*Zea mays*; NP_850017—*Arabidopsis thaliana*; AAA75104—*Triticum aestivum*; AAB07749—*Hordeum vulgare*, AAG23220—*Sorghum bicolor*; Q14011—*Homo sapiens*; AAH69219—*R. norvegicus*; JC6571—*Xenopus*

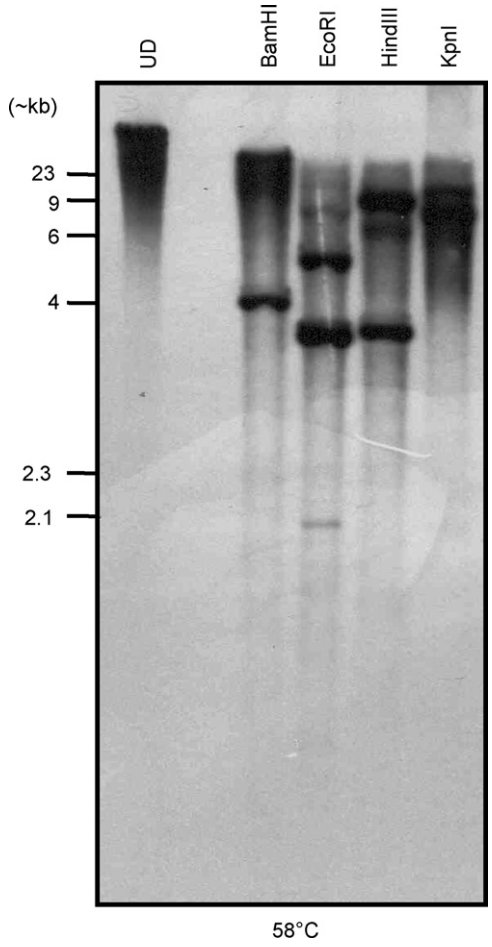

Fig. 2. Southern blot analysis of *Osgr-rbp4* gene. Ten micrograms of rice (P169) genomic DNA was digested with the indicated enzymes and processed for DNA blot analysis. The nylon filter was hybridized with the full-length Osgr-rbp4 cDNA at different stringency conditions (see text for details). The molecular weight markers are indicated at the left. UD: undigested DNA.

3.3. *Osgr-rbp4* transcript expression in rice

Expression analysis of *Osgr-rbp4* transcript was undertaken by Northern blotting. Significant level of *Osgr-rbp4* transcript was noted in uninduced seedlings (Fig. 3). Importantly, constitutive expression of *Osgr-rbp4* was found to be dependent on the time of sampling and showed circadian clock-dependent pattern in accumulation levels (Fig. 3). The dip noted in constitutive levels of *Osgr-rbp4* transcript was apparent at every 24 h time interval (see 8 a.m. sample time intervals at days 2–5 in Fig. 3). *Osgr-rbp4* mRNA was significantly up-regulated when seedlings were exposed to HS. The accumulation of *Osgr-rbp4* transcript was clearly noted in increased levels at 2 h, 4 h, 8 h and 24 h sampling intervals of HS at 35 °C and 42 °C (Fig. 4A and B). Increased *Osgr-rbp4* transcript accumulation was also noted after 2 h and 4 h of 45 °C stress (Fig. 4C). High temperature induced accumulation of *Osgr-rbp4* transcript was only observed at time points

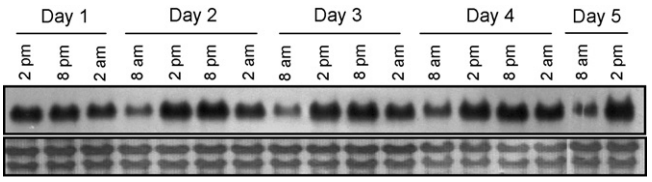

Fig. 3. Expression profiles of *Osgr-rbp4* at different time points (as shown at the top of the panel) in the day. RNA extracted from P169 rice seedlings was transferred onto nylon membrane and probed with full-length *Osgr-rbp4* cDNA. Methylene blue stained bands of rRNA in the lower panels depict similar amounts of RNA.

beyond 1 h in all the temperatures studied. *Osgr-rbp4* transcript showed slight up-regulation when seedlings were exposed for 72 h and 96 h of low temperature (LT) stress (Fig. 4D). *Osgr-rbp4* transcript was induced at low levels also by 1 h and 2 h of air-drying (AD) stress (Fig. 4D). Application of 200 mM NaCl (salt) to intact rice seedlings caused an increase in *Osgr-rbp4* transcript level notably at 72 h and 96 h of treatment (Fig. 4E). As compared to the unstressed controls, 2–3-fold higher transcript levels were detected after 2 h and 5 h of 100 mM DTT treatment (Fig. 4F).

3.4. OsGR-RBP4 protein expression in E. coli and rice

Osgr-rbp4 cDNA (full length) [*Osgr-rbp4* cDNA (FL)] was expressed in M15 *E. coli* cells. Following induction of cells by IPTG, over-expression of OsGR-RBP4 was clearly observed (data not shown). The corresponding protein was not visible in vector-transformed M15 cells or in uninduced M15 cells. OsGR-RBP4 was purified as almost a single band (as seen by CBB R-250 staining) using Ni-NTA agarose column. However, expression of this protein in *E. coli* appeared to slow down the growth of cells (data not shown). Next, truncated OsGR-RBP4 was expressed by transforming M15 cells with OsGR-RBP4ΔGD construct. Expressed protein in this case was expressed as ∼12 kDa protein on 15% SDS-gel. Purified truncated protein was dialyzed against 5% glycerol and then used for raising antibodies in mice.

Soluble proteins were isolated from unstressed and HS rice tissues. Western blotting analysis showed that OsGR-RBP4 was constitutively expressed under unstressed conditions in rice shoots (Fig. 5I, upper panel). OsGR-RBP4 protein did not show circadian rhythmicity in accumulation pattern (Fig. 5I, upper panel) as was observed for the transcript (Fig. 3). In response to HS, OsGR-RBP4 protein levels significantly declined. The reduction in the protein levels began within the first 3 h of stress treatment and almost no protein was detected on the immunoblots after 6 h of continued stress (Fig. 5I, lower panel). OsGR-RBP4 was noted to be rapidly lost within 1 h of 100 mM DTT treatment to seedlings (Fig. 5II). Unlike HS, other stress conditions (i.e. LT, AD and salt) did not show pronounced effect on the levels of OsGR-RBP4 (Fig. 5III).

laevis. Sequences were aligned by Clustal-V method in the MEGALIGN module of DNAStar. Shaded amino acids represent the conserved residues. The two conserved RNA binding consensus sequences and the GGYGG boxes have been enclosed in boxes. (D) Phylogenetic relationship of the proteins was determined using the CLUSTAL-V of DNAStar. Other details are same as in Panel A.

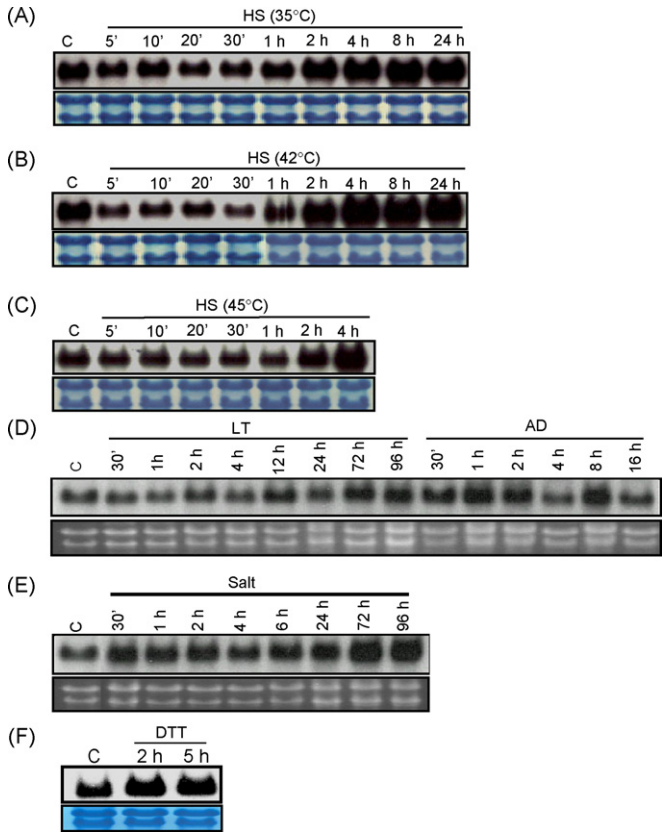

Fig. 4. Expression profiles of *Osgr-rbp4* in response to different stresses. RNA extracted from seedlings (A–E: P169; F: PB1) given high temperature (A–C), low temperature (D), air-drying (D), salt (E) and DTT (F) stresses, was transferred onto nylon membrane and probed with full-length *Osgr-rbp4* cDNA. Methylene blue stained bands of rRNA depict similar amounts of RNA. HS: heat shock; LT: low temperature; AD: air-drying. Salt: 200 mM NaCl; DTT: 100 mM DTT.

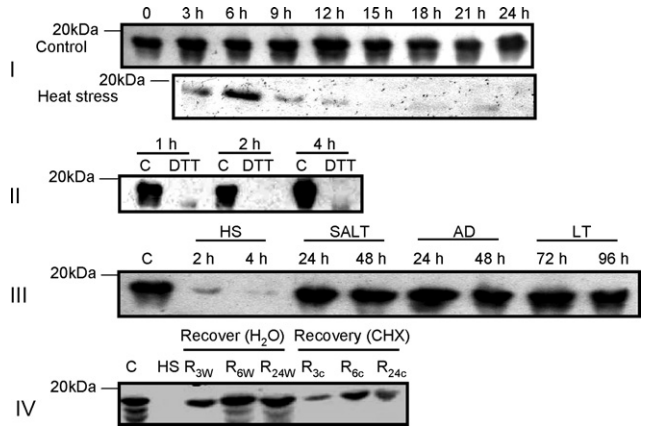

Fig. 5. Western analysis of OsGR-RBP4. (i) Thirty micrograms of total soluble rice (PB1) proteins isolated from seedlings under unstressed control (upper panel) and continuous heat stress (lower panel). (ii) Thirty micrograms of total soluble proteins isolated from PB1 control (C) and 100 mM DTT treated seedlings. (iii) Thirty micrograms of total soluble proteins isolated from PB1 seedlings harvested at different time durations (as indicated at the top) at HS (45 °C), salt, AD (air-drying) and LT (low temperature). (iv) Thirty micrograms of total soluble proteins isolated from PB1 seedlings heat stressed at 45 °C for 4 h and then placed for recovery at room temperature (RT) either in water or in presence of CHX (50 μg ml^{-1}). C—control sample at the time of heat stress; HS—sample harvested immediately after HS. R_{3W}, R_{6W}, R_{24W}-recovery samples harvested after 3 h, 6 h or 24 h in water. R_{3c}, R_{6c}, R_{24c}-recovery samples in the presence of CHX. Nitrocellulose membrane was first probed with polyclonal antisera raised against OsGR-RBP4 (1:1000) and subsequently with HRP-conjugated anti-mice secondary antibodies (1:1000). Blots were processed for enhanced chemiluminescence. HS: heat stress; salt: 200 mM NaCl; AD: air-drying; LT: low temperature.

Expression levels of OsGR-RBP4 during recovery from HS (45 °C HS given for 4 h) was further examined. Recovery treatment was carried out at room temperature for 3 h, 6 h and 24 h, while placing seedlings either in presence of cycloheximide (CHX; 50 μg ml^{-1}) or water (W). Proteins cross-reacting with anti-OsGR-RBP4 antibodies were resolved in three bands upon longer incubation with developer in Western blotting (the uppermost being the major band). OsGR-RBP4 was not detected in heat-stressed tissues (Fig. 5IV, lane HS). During recovery in water, the major OsGR-RBP4 band was regained to almost its constitutive amounts within 3 h (Fig. 5IV, lane R_{3W}). At 6 h of recovery, all the three bands were seen. The major OsGR-RBP4 protein was also recovered to an extent in 3 h sample placed for recovery in CHX solution. However, two minor bands were not apparent even at 24 h of recovery in CHX-treated seedlings (Fig. 5IV, lane R_{24c}).

3.5. Thermotolerance assays using yeast cells expressing *OsGR-RBP4*

OsGR-RBP4 (FL) construct was cloned in pGV8 vector. Colonies that grew on the minimal SD media were tested for the presence of respective transcripts.

The ability of OsGR-RBP4 in improving the basal thermotolerance in yeast [FY3 (wild type), FY3-pGV8 (WT cells transformed with vector backbone) and FY3-*Osgr-rbp4* (FL) (WT cells transformed with full-length *Osgr-rbp4*)] was tested by exposing cells to heat stress at 50 °C for 30 min (without 37 °C pre-treatment step). Yeast cells over-expressing the OsGR-RBP4 could survive lethal stress of 50 °C for 30 min. In contrast, the WT and the vector alone cells showed reduced viability during recovery following the lethal HS (Fig. 6).

3.6. Cellular localization of *OsGR-RBP4*

Immunolocalization analysis of OsGR-RBP4 was carried out *in situ*, in rice leaf sections, rice mesophyll protoplasts and BY2 tobacco protoplasts (using anti-OsGR-RBP4 antibodies raised in this study and commercially obtained Alexa Fluor488

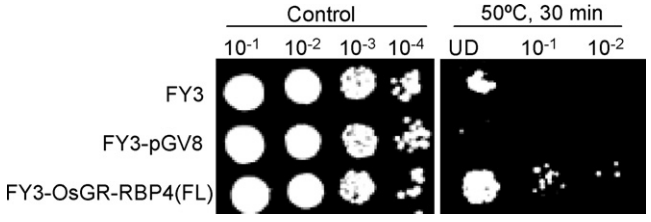

Fig. 6. Genetic transformation of yeast cells with OsGR-RBP4. Complementation assay of WT yeast cells with *Osgr-rbp4*. Different yeast strains used in this assay are indicated at the left. Assay conditions are shown on top of the panel. UD: undiluted yeast culture. See text for the details of the strains used.

labeled anti-mice secondary antibodies). Control (unstressed) rice leaf sections (Fig. 7A(c)) showed fluorescence several folds more than the negative control (panel a; representing rice section probed with only the secondary antibodies). Most of the

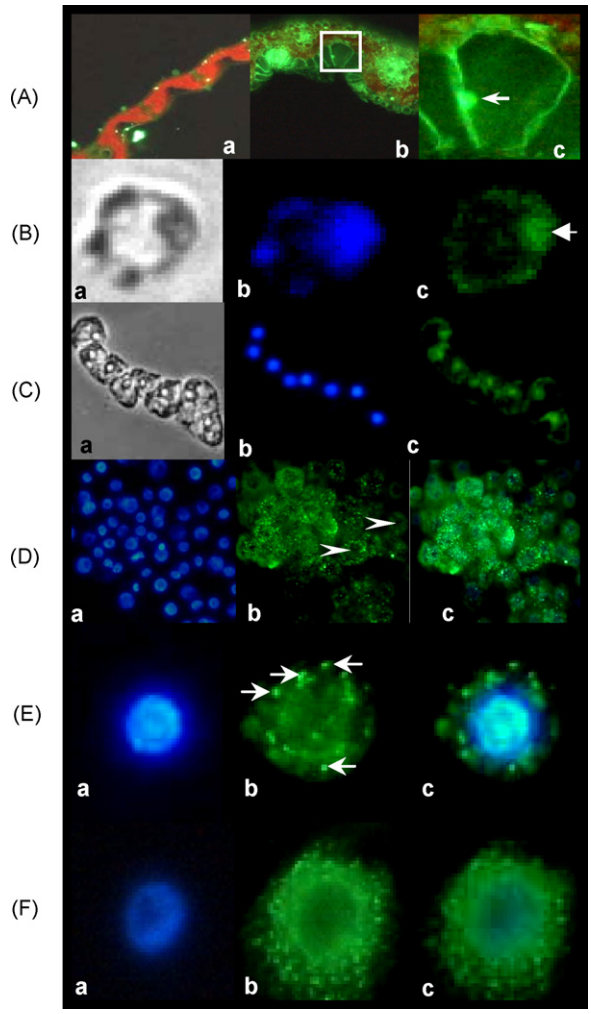

Fig. 7. Localization of OsGR-RBP4 in rice leaf sections (A), rice mesophyll protoplasts (B) and tobacco BY2 cells (C–F). Primary and secondary probings were carried out using anti-OsGR-RBP4 (dilution 1:200) and Alexa Fluor488 conjugated anti-mice secondary antibodies (dilution 1:200), respectively. (A): (a) Rice leaf sections probed with only the secondary antibodies. (b) OsGR-RBP4 localization in unstressed control rice leaf tissues. (c) Magnified portion of the region (shown in box) from panel b. Nuclear region in panel c is shown by an arrow. (B): (a) Rice mesophyll protoplasts (under uninduced control conditions) viewed in bright field. (b) DAPI-stained protoplasts. (c) Protoplasts probed with both primary and secondary antibodies as of above. Nuclear region in panel c is shown by arrow. (C): (a) Tobacco BY2 protoplasts (under uninduced control conditions) viewed in bright field. (b) DAPI-stained protoplasts. (c) Protoplasts probed with both primary and secondary antibodies as of above. (D): (a) Tobacco BY2 DAPI-stained protoplasts (following 42 °C, 1 h heat stress). (b) Protoplasts probed with both primary and secondary antibodies as of above (arrowheads mark the nuclear regions). (c) Merged image of panels a and b. (E): (a) Magnified view of a single protoplast from D(a) panel. (b) Magnified view of a single protoplast from D(b) panel. (c) Magnified view of a single protoplast from D(c) panel. (F): (a) Magnified view of a single tobacco BY2 protoplast (following 42 °C, 1 h heat stress) viewed after DAPI-staining. (b) Protoplast probed with both anti-OsHsp18 primary antibodies and Alexa Fluor488 conjugated anti-mice secondary antibodies. (c) Merged image of panels a and b.

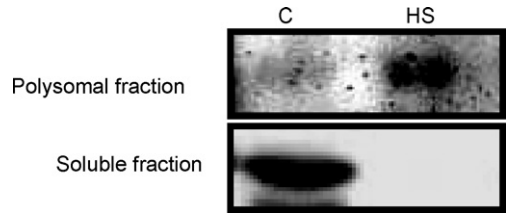

Fig. 8. Analysis of OsGR-RBP4 in rice polysomal fraction. Polysomal (upper panel) and soluble (lower panel) protein extracts were first resolved on 15% SDS-gel and then electro-blotted to nitrocellulose membrane. The membrane was probed with anti-OsGR-RBP4 antibodies. C: uninduced control seedlings; HS: heat stressed tissues (42 °C, 3 h).

fluorescence was noted to emanate from the nuclear region (shown by arrow in Fig. 7A(c)). Analysis of rice mesophyll protoplasts also showed that OsGR-RBP4 protein is mainly in nucleus as DAPI staining (Fig. 7B(b)) and fluorescence showing (Fig. 7B(c)) regions showed an overlap. Likewise in BY2 protoplasts, most of the fluorescence corresponding to OsGR-RBP4 was detected in the nucleus under unstressed control conditions (Fig. 7C(b and c)). In heat shocked tobacco BY2 cells, fluorescence output was mainly detected in the cytoplasm (Fig. 7D). The nucleus as seen by DAPI staining (Fig. 7D(a)) appeared as hollow structure in fluorescence yield (Fig. 7D(b)). In fact, fluorescence was noted to be sequestered into discrete cytoplasmic foci in heat-shocked BY2 cells (Fig. 7E(b); marked with arrows). As a control reaction, anti-HSP18 antibodies were employed to detect HSP18 protein under same experimantal conditions. HSP18 was noted to be concentrated into cytoplasmic granules while nucleus appeared hollow structure during HS (Fig. 7F(b and c)).

3.7. OsGR-RBP4 is localized in polysomal protein fraction following HS

Total polysomal protein fraction isolated from unstressed control and HS rice seedlings was analyzed for OsGR-RBP4 by Western blotting. OsGR-RBP4 was barely present in this fraction under unstressed control conditions. However, OsGR-RBP4 protein appeared in significantly higher levels in polysomal fraction under conditions of HS (Fig. 8, upper panel). In the same experiment, Western analysis using total soluble protein was carried out. Under control conditions, high expression of OsGR-RBP4 was detected in the soluble fraction of the control sample while the corresponding protein band was not detected in the soluble fraction corresponding to heat-stressed sample (Fig. 8, lower panel).

4. Discussion

In the course of differential screening of rice heat cDNA library, we isolated a cDNA clone whose nucleotide sequence to a large extent matched with GR-RBPs reported in rice as well as in other plants. Deduced amino acid sequence showed high degree of similarity and identity with previously reported rice GR-RBPs (Fig. 1A). At its N-terminus, it has RRM composing of RNPI and RNPII (Fig. 1C). It has a GD domain composing of RGG and GGYGG boxes at its C-terminus (Fig. 1C). As there

are already three full-length rice GR-RBPs cDNA sequences submitted in NCBI database (with accession numbers AF010579, AJ002893 and AJ002894), we have assigned *Osgr-rbp4* nomenclature to the full-length *Osgr-rbp* cDNA isolated in this study. Based on detailed *in silico* analysis undertaken in this study using NCBI (http://www.ncbi.nlm.nih.gov/) and KOME (http://cdna01.dna.affrc.go.jp/cDNA/) database, we propose that there are 13 members in all, in rice OsGR-RBP family (AF010579, AJ002893, AJ002894, AJ302060, AK058443, AK059164, AK064923, AK070016, AK072673, AK103069, AK111046, AK119238 and AK119242; Supplementary data 2). It is important to indicate here that detailed functional characterization of none of these proteins has been made as yet. To answer how complex is the gene family that encodes OsGR-RBP4 in rice, Southern analysis of genomic DNA was carried out. This work was initially done maintaining 42 °C as the hybridization temperature. However, the blot showed only a smeared pattern. Similar was the case when hybridization temperature was raised to 50 °C. As the probe used in this study corresponded to full-length *Osgr-rbp4* cDNA sequence, we believe that RNA binding domain sequences present in one or more copies in diverse RBPs [7], are leading to this complexity in blots. When hybridization temperature was further increased to 58 °C, only three to four cross-reacting bands lighted up (Fig. 2). These species of bands possibly represent the genes which are closer to *Osgr-rbp4* sequence obtained in this study.

In *Arabidopsis*, *grp* transcripts are noted to follow clock-dependent pattern [51]. Levels of *Osgr-rbp4* transcripts were clearly modulated by circadian rhythmicity when monitored for four consecutive days at 6 h interval (Fig. 3). Lowest levels of *Osgr-rbp4* transcript were observed in samples harvested at 8 a.m. and the highest levels were observed in samples harvested at 2 p.m., on a daily basis. Transcript accumulation of the gene corresponding to *A. thaliana* RNA binding protein AtGrp7 is shown to occur preferentially at the end of the daily light period [52]. Recently, Raab et al. [53] have observed that RNA binding protein encoding *CpRNP29* gene also follows circadian rhythmicity. Most of the circadian clock-regulated genes are noted to be involved in light absorption and carbon metabolism [3]. There is no evidence as yet whether GR-RBPs have any direct or auxiliary role in such basic cellular functions. On the basis of the fact that RBP transcripts are shown to be cold-inducible in several instances, these proteins have been implicated in low temperature stress response [3,54]. *Osgr-rbp4* transcript was noted to be marginally up-regulated by low temperature in this study (at 72 h and 96 h of sampling; Fig. 4). In contrast to low temperature, *Osgr-rbp4* transcript showed higher induction in HS samples (Fig. 4). In case of HS, 2–4 h interval appeared sufficient enough to elicit high transcript accumulation. There is not much data showing whether plant GR-RBPs have any role in high temperature response but RBPs are reported to be critical for survival at high temperature in yeast [55]. When full-length OsGR-RBP4 was expressed in FY3 wild type yeast cells, recombinant cells showed increased basal higher temperature tolerance as compared to WT FY3 cells or cells transformed with vector backbone (Fig. 6).

Heat stress is known to increase the stability of specific mRNAs [56]. Noon et al. [57] reported that post-transcriptional modifications in tRNAs and rRNAs are especially abundant in thermophilic organisms and these modifications appear to play a functional role in structural stabilization of RNA at elevated temperatures. It is possible that OsGR-RBP4 provides increased high temperature protection to yeast cells through its binding to mRNA molecules. Next question we asked was where in cytoplasm is OsGR-RBP4 localized? Immunoblot analysis showed that OsGR-RBP4 was apparently lost from the soluble protein fraction during the course of high temperature stress (Fig. 5). The observation on high temperature induced loss of OsGR-RBP4 protein is in contrast to what we observed for the *Osgr-rbp4* transcript (Fig. 6). We inferred that the OsGR-RBP4 protein is undergoing either high turnover rates or is changing its localization site (or both) such that it is no more a component of the soluble protein fraction. When rice seedlings (placed in distilled water) were allowed to recover from the HS stress treatment, OsGR-RBP4 protein was recovered within 3 h (Fig. 4). Importantly, an appreciable recovery in the major cross-reacting OsGR-RBP4 was noted when seedlings were allowed to recover for 3 h in cycloheximide (CHX) solution rather than water. We infer from this observation that OsGR-RBP4 noted during recovery period may not have been *de novo* synthesized. Rather, it represents protein that changed its localization during the stress regime. There are clear hints in literature on mammalian cells that specific RRM containing proteins are relocated into discrete foci called stress granules (SGs; representing mainly the polysomal fraction) in a stress-dependent manner [58]. Polysomes and SGs are considered to be dynamic cytoplasmic structures which are in constant equilibrium in a cell under stress [58]. SGs have been shown to be present in tomato protoplasts [59]. GR-RBPs in selected instances have been noted to be nuclear-localized [60]. Shinozuka et al. [61] have reported that *Lolium perenne LpGRP1* is a nuclear localized protein. Immunofluorescence analysis done with the help of Alexa Fluor488 conjugated anti-mice secondary antibodies in our study showed that OsGR-RBP4 is detectable mainly in the nucleus in unstressed control cells of rice leaf sections as well as in rice mesophyll and tobacco BY2 protoplasts (Fig. 7). It was further seen that this protein is detected mainly in the cytoplasm of the heat shocked tobacco BY2 cells (Fig. 7). In heat-shocked BY2 protoplasts, fluorescence accruing from the cytoplasmic fraction was noted to be sequestered into discrete cytoplasmic foci. These phase-dense particles may represent SGs. Next, polysome fraction proteins isolated from uninduced control and HS stressed rice seedlings were analyzed by Western blotting. Under unstressed control conditions, OsGR-RBP4 was conspicuously seen in the soluble protein fraction but was not detected in the polysomal protein fraction of the seedlings. However, OsGR-RBP4 protein was present in the polysomal fraction of the HS seedlings (Fig. 8). From the above account, it emerges that OsGR-RBP4 protein present in soluble protein fraction (representing possibly cytoplasmic and nuclear proteins) in control seedlings shuttles to polysomal fraction in heat shocked seedlings. Human HnRNP18A protein is previously shown to

be a GR protein with 2× RRM. This protein is shown to be exported out of the nucleus only if it is bound to mRNAs [62]. Through such experiments, it has emerged that RNA binding, mRNA export and formation of SGs are interconnected phenomena. According to Kedersha and Anderson [58], SGs are dynamic ribonucleoprotein complexes containing mRNAs. Constitutively expressed yeast HRP1 protein is exported from the nucleus following a hyper-osmotic shock [6]. It has been hypothesized that proteins like yeast HRP1 have housekeeping function(s) under control conditions when inside the nucleus and specific regulatory roles in the cytoplasm during stress periods [63]. This example appears analogous to observations made in this study. Specific yeast mutants have been isolated which have aberrant cytoplasmic transport of HS mRNAs [55]. Analysis of such mutants shows that major cytoplasmic shuttling proteins are important for stress-dependent export of selective mRNAs from the nucleus. This takes place with concurrent cessation in the export of housekeeping mRNAs [64]. OsGR-RBP4 was found to be constitutively present in nuclear compartment under unstressed control conditions and was rapidly exported to the cytoplasm after HS. It is possible that under normal conditions, OsGR-RBP4 is involved in housekeeping functions like splicing and ribosome biogenesis. During stress, OsGR-RBP4 may be involved in more specific stress-associated roles like regulating RNA export, stability or translation.

RBPs are shown to be involved in diverse stresses. Heteronuclear ribonucleoprotein RBPA18 is induced and translocated from the nucleus to the cytoplasm after UV radiation. Czyzyk-Krezeska et al. [65] implicated RBPs in transcript stabilization during hypoxia and extracellular signals. An important cellular response to high temperature, hypoxia or oxidative stress is production of unfolded or misfolded polypeptides inside cells [66]. This often results into a cellular state called the ''unfolded protein response'' (UPR). UPR has been extensively worked out in mammalian cells and has been found to be accompanied by induction of several genes including chaperones, transcription factors and members of the translational initiation and RNA metabolic pathways [67]. The plant cells are shown to demonstrate UPR when treated with agents like dithiothreitol (DTT; [68]). In this study, OsGR-RBP4 protein was rapidly lost from the soluble protein fraction in response to DTT treatment. This effect was similar to what we noted when HS stress was imposed to seedlings. In mammalian cells, DTT is used to impose oxidative stress [58]. Based on this account, we speculate that UPR is associated with SG formation in plants as well.

Selective translation noted for stress-associated genes is considered to be due to different RBPs, translation initiation factors and other accessory factors [23,24]. We propose that OsGR-RBP4 might be involved with selective translation and transcript stability under stress conditions. Recently, *Arabidopsis* plants over-expressing AtRZ-1a gene (encoding for GR-RBP, driven by CaMV35S promoter) have been raised [69,70]. The transgenic *Arabidopsis* plants that over-express this gene displayed earlier germination and better seedling growth than the WT under low temperature conditions. Moreover, AtRZ-1a over-expressing transgenic *Arabidopsis* plants were more freezing tolerant than the WT plants. This group used Zn-finger containing GR-RBP which is low temperature inducible in their work. On the other hand, transgenic *Arabidopsis* plants over-expressing AtGR-RBP4 did not show any significant changes in cold or freezing tolerance as compared to the wild type [71]. OsGR-RBP4 noted in this study does not have signatures for Zn binding and is up-regulated by high temperature stress. It would be important to examine stress response of plants raised with altered levels of OsGR-RBP4 for under- or over-expression in future attempts.

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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.plantsci.2007.04.010.

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