Characterization of 5'UTR of rice ClpB-C/Hsp100 gene: evidence of its involvement in post-transcriptional regulation

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Abstract Rice (*Oryza sativa*) ClpB-C (OsClpB-C) protein is expressed upon heat stress in vegetative tissues and constitutively in seeds. We produced stably transformed *Arabidopsis* plants carrying β -glucuronidase (Gus) reporter gene downstream to 1-kb OsClpB-C promoter (1kbPro plants). In the 1kbPro plants, expression of Gus transcript and protein followed the expression pattern of OsClpB-C gene in rice plants, i.e., heat induced in vegetative tissues and constitutive in seeds. Next, we produced transgenic *Arabidopsis* plants containing Gus downstream to 862-bp fragment of OsClpB-C promoter [lacking 138 nucleotides from 3' end of the 5' untranslated region (5'UTR); Δ UTR plants]. In Δ UTR plants, Gus transcript was expressed in heat-inducible manner, but strikingly, Gus protein levels were negligible after heat treatment. However, Gus protein was expressed in Δ UTR seedlings at levels comparable to 1kbPro seedlings when recovery treatment of 22 °C/2 h was given post heat stress (38 °C/15 min). This suggests that 5'UTR of OsClpB-C gene is involved in its post-transcriptional regulation and is an obligate requirement for protein expression during persistent heat stress. Furthermore, the Gus transcript levels were higher in the polysomal RNA fraction in heat-stressed seedlings of 1kbPro plants as compared to Δ UTR plants, indicating that 5'UTR aids in assembly of ribosomes onto the Gus transcript during heat stress. Unlike the case of seedlings, Gus protein

was formed constitutively in Δ UTR seeds at levels comparable to 1kbPro seeds. Hence, the function of 5'UTR of OsClpB-C is dispensable for expression in seeds.

Keywords *Arabidopsis* · ClpB-C/Hsp100 · Heat stress · Rice · Seed · 5'UTR · Vegetative tissues

Introduction

Living cells respond to heat stress (HS) by resorting to two important adaptive changes in gene expression: (a) messenger RNAs (mRNAs) encoding for heat shock proteins (Hsps) are predominantly transcribed, and (b) Hsps are preferentially synthesized at a time point when the translation of most other cellular mRNAs is largely suppressed (de Nadal et al. 2011). These changes enable cells to meet the sudden increased demand of Hsps to mitigate the damage caused by HS. The transcriptional induction of Hsp promoters in plants is mainly regulated by heat shock factors (Hsfs), which undergo trimerization and activation during HS (Qu et al. 2013). Hsfs bind to well-defined and highly conserved heat shock element (HSE) sequences and facilitate increased transcription of Hsp genes. Eukaryotic HSEs are adjacent and inverse repeats of the core unit 5'-nGAAn-3' that forms the binding sites for the Hsfs (Fragkostefanakis et al. 2014; Mittal et al. 2011).

In general, the translation initiation process starts with the binding of the initiation factor eIF4E to the 7-methyl guanosine cap structure at the 5' end of the mRNAs. The eIF4E interacts with the platform protein eIF4G forming eIF4F complex, which directs the binding of 40S subunit of the ribosome. The resulting complex then scans the 5'untranslated region (UTR) of the mRNA molecules before reaching the translation initiation codon AUG (Holcik and Pestova 2007). This scanning process is greatly influenced by the features of

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the 5'UTR. Evidently thermodynamically stable RNA secondary structure present in the 5'UTR impairs scanning: the 5' UTRs with weak potential secondary structures have an advantage in ribosome recruitment over those with a higher potential for secondary structure (Kawaguchi and Bailey-Serres 2005). The onset of HS results in reduced phosphorylation of eIF4E which inactivates the 5' cap recognition by this complex, thereby hampering the overall cap-dependent translation activities (Echevarria-Zomeno et al. 2013). Under such conditions, mRNAs encoding proteins essential for cell survival bypass cap-dependent translation adopting alternative means (Martinez-Salas et al. 2012). In mouse, 5'UTR of histone mRNA contains two structural elements that sequester the cap and enable cap-independent binding to eIF4E, thereby loading the 43S complex near the AUG codon (Martin et al. 2011). In some special circumstances where 5' UTRs are lengthy with complex secondary structures like mRNAs encoding proto-oncogenes, growth factors, and homeodomain proteins, cells deploy an alternative mechanism of translation initiation involving an internal ribosomal entry site (IRES) (Liu et al. 2012). IRES is reported in majority of RNA viruses where it has evolved as a mechanism to recruit translation machinery in a more efficient and effective way, close to the initiation codon, to overcome the translational inhibition from stressed host cell (Echevarria-Zomeno et al. 2013). In case of Hsp mRNAs, the UTRs play a crucial role in the post-transcriptional regulation (Lawless et al. 2009). Importantly, the synthesis of selective Hsps (i.e., Hsp90, Hsp70, Hsp60, and Hsp27) remains unaffected in cells with reduced eIF4E, indicating that Hsp mRNAs have little or no requirement for cap recognition mechanism (DeGracia et al. 2008). It is suggested that these mRNAs may use an alternative mechanism for translation initiation by employing their 5'UTRs.

Caseinolytic protease (Clp) family is constituted of AAA⁺ (ATPases associated with diverse cellular activities) superfamily members having the protein disaggregation ability. ClpB (Hsp100 protein) is essentially involved in non-degradative pathway that brings about renaturation of heat-aggregated proteins (Mishra and Grover 2015). It thus constitutes an essential component of cells under HS. ClpBs are conserved in bacteria, yeast, and plants (Singh and Grover 2010; Singh et al. 2010). Plants contain three homologs of ClpB proteins: cytoplasmic/nuclear ClpB (ClpB-C), mitochondrial (ClpB-M), and chloroplastic (ClpB-P) (Lee et al. 2007b; Singh et al. 2010). Acquisition of thermotolerance in plants is particularly attributed to ClpB-C proteins, which are expressed at a higher titer in vegetative tissues during HS (Hong and Vierling 2000, 2001; Singh and Grover 2010). Hsps are constitutively expressed during reproductive stages besides their characteristic heat-inducible expression in vegetative tissues (Crone et al. 2001; Sarkar et al. 2009; Young et al. 2001). In *Arabidopsis*, ClpB-C is accumulated in seeds (Hong and Vierling 2001). Singla et al. (1998) reported that the

constitutive levels of ClpB-C protein were in particular high in developing and mature rice grains. Singh et al. (2012) showed that 2-kb OsClpB-C promoter expresses β -glucuronidase (Gus) constitutively in embryonal half of the seeds in stably transformed rice plants. It has further been worked out that HS-induced up-regulation of OsClpB-C transcript is mediated by interaction of OsHsfA2C with the single functional HSE marked by “gaatattcat” sequence in OsClpB-C promoter (Mittal et al. 2011; Singh et al. 2012). While the studies on expression of plant ClpB-Cs under HS have gained much attention, the regulation of ClpB-C expression during reproductive stages remains largely unexplored (Mishra and Grover 2015). We herein report analysis on post-transcriptional regulation of OsClpB-C in context of the role of 5'UTR toward its constitutive expression in seeds and HS-induced expression in vegetative tissues. The data presented was obtained by stably transforming OsClpB-C gene promoter, with full as well as truncated 5'UTR, driving Gus reporter gene in *Arabidopsis* plants. We noted that 5'UTR of OsClpB-C aids in loading of transcripts onto polysomes, resulting in an uninterrupted and enhanced translation during HS. Importantly, we found that 5'UTR is an obligate requirement for ClpB-C expression during HS but dispensable for its constitutive expression in seeds.

Materials and methods

Plant material and growth conditions

Arabidopsis seeds were placed in pots containing soilrite. The seeds were stratified by keeping the pots in the dark at 4 °C for 48–72 h. The pots were placed in culture room maintained at 22±1 °C with 16:8-h light and dark regimes with a light intensity of 100–125 $\mu\text{mol m}^{-2} \text{s}^{-1}$ for raising *Arabidopsis* rosettes. For *Arabidopsis* tissue culture, seeds were surface sterilized with 70 % ethanol for 30 s followed by a treatment of 2 % (v/v) sodium hypochlorite with one drop of Tween-20 for 5 min and washed with sterile water three to four times and finally suspended in 0.1 % (w/v) agar. The seeds were plated onto Murashige-Skoog (MS) medium containing 1 % sucrose and 0.8 % agar (pH 5.8) in petri plates, stratified, and finally shifted to culture room. For selecting transformants, surface-sterilized seeds were plated on ½ MS medium plates supplemented with 50 mg L⁻¹ of kanamycin and 150 mg L⁻¹ of augmentin. Various genetically transformed *Arabidopsis* lines were transferred to pots in culture room for further maturation. For raising rice [*Oryza sativa* L. cultivar Pusa Basmati 1 (PB1)] seedling, seeds were surface-sterilized using 0.1 % HgCl₂ solution and rinsed several times with sterilized water. Surface-sterilized seeds were placed on wet cotton for germination in the dark for 2 days and then in light for varied periods under culture room conditions. Ten-day-old seedlings

were placed in pots containing soil and shifted to green house for further growth.

Plasmid construction for plant transformation

For studying the genetic regulation of OsClpB-C promoter, three different promoter fragments, 2 kb (2kbOsClpB-Cpro) and 1 kb (1kbOsClpB-Cpro) upstream of ATG codon and 862 bp (Δ UTRClpB-Cpro; excluding 138 nt of 5'UTR from the 3' end of the 1kbOsClpB-Cpro), were PCR amplified from PB1 rice genomic DNA and cloned upstream to Gus in between HindIII and XhoI of pBT2Gus vector. The 2kbOsClpB-Cpro:Gus, 1kbOsClpB-Cpro:Gus, and Δ UTRClpB-Cpro:Gus fragments were cleaved from the resulting plasmids and introduced in pCAMBIA2300 binary vector. The resulting binary plasmids were introduced in AGL-1 strain of *Agrobacterium tumefaciens* and stably transformed in *Arabidopsis*, Col-0 ecotype (giving rise to 2kbPro, 1kbPro, and Δ UTR plants, respectively). Plant transformation was carried out employing floral dip method as described by Clough and Bent (1998). For each construct, single round of transformation (floral dip) was performed to obtain a pool of transformed as well as untransformed T1 seeds. Seeds were plated on antibiotic (kanamycin) containing medium for transgenic screening. Several transformed (kanamycin resistant) T1 plants were obtained for each construct, and T2 seeds were harvested. T2 seeds from several lines were again plated on kanamycin-containing media for segregation analysis. Out of multiple lines, three with T2 germination frequencies conforming 3:1 segregation ratio (based on the screening of ~80–90 seeds) were selected for homozygous screening. Several individual plants for each of these three T2 lines were grown, and T3 seeds were harvested. Among the individuals for each of the three lines, the ones showing T3 germination frequency of ~100 % in next round of kanamycin selection (based on the screening of ~100 seeds) were chosen as homozygous for experimental analysis. Homozygous lines were further confirmed for the status of transfer DNA (T-DNA) introgression through appropriate PCRs (data not shown). Experiments were performed in T4 generation (homozygous plants). Further, for each analysis, seedlings were first raised on kanamycin selection to ensure homozygosity (~100 % germination).

For making CaMV35S-5'UTR-GFP construct, 138-bp UTR fragment was amplified using BamHI and cloned upstream to green fluorescent protein (GFP) gene in BamHI-digested pSM-GFP vector. The CaMV35S-GFP (pSM-GFP vector) construct was used as control. The two constructs were mobilized in HindIII- and EcoRI-digested pCAMBIA1300 binary vector for rice transformation. Rice transformation protocol was as employed earlier in our laboratory (Katiyar-Agarwal et al. 2003). Genomic DNA was isolated from transgenic plants employing protocol described by Kobayashi et al.

(1998). For confirmation of T-DNA integration, appropriate PCR assays were performed using specific primers. Various primers used in this study are listed in Table S1.

Constructs for transient assay

To analyze the 138 nt of 5'UTR for cryptic promoter activity, this fragment was cloned in HindIII and BamHI sites of pSM-GFP vector (construct 5'UTR-GFP). This construct along with controls (pSM-GFP; construct CaMV35S-GFP) was analyzed by transient expression in onion epidermal peel. Biolistic PDS-1000/He particle delivery system (Bio-Rad, USA) was used for DNA delivery (Lee et al. 2008). The plates were incubated in the dark for 10–12 h at 28 °C. The onion peels were examined in TCS SP5 laser scanning confocal microscope (Leica, Germany) for fluorescence detection. For GFP analysis, 488-nm laser was used for excitation and signals were detected between 505 and 550 nm.

Gus expression analysis

Histochemical analysis of Gus activity was carried out using protocol described by Jefferson et al. (1987). 5-Bromo-4-chloro-3-indolyl- β -D-glucuronic acid (X-gluc) was used as a substrate for qualitative estimation of Gus activity. Control tissues (10-day-old seedlings, leaves from a month-old mature plants, inflorescence, siliques, and seeds) were stained overnight at 37 °C. For HS treatment, seedlings were first exposed to 38 °C for requisite time periods and then stained overnight at 37 °C. For each construct/transgenic, assays were performed with the three independent homozygous lines. For metal stress, seedlings were floated in solutions of requisite salts of metals for 6 h and stained as above. After Gus staining, chlorophyll was leached from the tissues with ethanol/acetic acid (3:1). The transgenic lines, positive for histochemical Gus staining, were analyzed quantitatively by fluorimetric analysis as described by Jefferson et al. (1987). Soluble protein from *Arabidopsis* seedlings and seeds was extracted in Gus extraction buffer (50 mM sodium phosphate buffer, pH 7.0, 10 mM EDTA, pH 8.0, 10 mM β -mercaptoethanol, 0.1 % Triton X-100, 0.1 % sarcosine) and quantified using standard Bradford's method. Reactions were set using equal quantities of protein in Gus assay buffer, which comprises 2 mM 4-methylumbelliferyl- β -D-glucuronide (MUG) in Gus extraction buffer. After an incubation of 15 h at 37 °C, reaction was stopped by adding 0.2 M Na₂CO₃. Fluorescence of 4-methylumbelliferone (4MU; the reaction product obtained through activity of Gus on MUG) was measured by using DyNA Quant TM 200 fluorimeter (Hoefer Pharmacia Biotech Inc., CA, USA). For each construct/transgenic, fluorimetric analysis was performed separately for the three individual homozygous lines. For quantitative estimation of Gus activity in seedlings, a pool of ten seedlings was taken in each

biological sample whereas in case of seeds, roughly equal number of seeds (corresponding to equal quantity in mg) was taken. Each fluorometric experiment was performed thrice, and similar results were obtained. Data presented in the results are from one representative experiment.

RNA isolation, cDNA synthesis, and sqRT-PCR analysis

Total RNA from seeds at different stages of germination was isolated in equal volumes of extraction buffer [0.4 M LiCl, 0.2 M Tris, pH 8, 25 mM EDTA, and 1 % (w/v) sodium dodecyl sulfate (SDS)] and chloroform (550 μ l). After centrifugation, supernatant was transferred to a new microcentrifuge tube (MCT) and 500 μ l of water-saturated acidic phenol was added. Along with phenol, 200 μ l of chloroform was added and mixture was centrifuged for 3 min at max speed (at 4 °C). In a new MCT, one third volume of LiCl was added to the supernatant and kept at -20 °C for 0.5 h for precipitation. Resulting pellet was dissolved in ddH₂O and treated with DNase I. Following this, carbohydrate was precipitated by adding 470 μ l of ddH₂O, 7 μ l 3 M CH₃COONa, pH 5.2, and 250 μ l of absolute ethanol in the sample. After centrifugation at 4 °C for 10 min, supernatant was transferred in a fresh MCT and 43 μ l 3 M CH₃COONa, pH 5.2, and 750 μ l absolute ethanol was added and kept at -20 °C for 1 h. Resulting pellet was washed in 70 % (v/v) ethanol. After washing, pellet was resuspended in ddH₂O. Total RNA from the control and stressed *Arabidopsis* seedlings was isolated in GITC extraction buffer (Chomczynski and Sacchi 1987). For the synthesis of first-strand complementary DNA (cDNA), 2 μ g of total RNA was reverse transcribed using M-MuLV reverse transcriptase (MBI Fermentas, Lithuania). For removal of genomic DNA contamination, DNase I treatment was given. For semiquantitative reverse transcriptase PCR (sqRT-PCR) of Gus transcript, amplification parameters were standardized. Actin was amplified as an internal control for the normalization of mRNA levels in different cDNA samples.

Analysis of association of Gus mRNA with polysomes

Polysomal RNA was isolated as described by Taliercio and Ray (2001). Approximately 5 g of tissue (whole *Arabidopsis* seedlings) was ground in liquid nitrogen and suspended in 5 ml grinding buffer [0.2 M Tris (pH 9.0), 0.4 M KCl, 0.06 M Mg acetate, 0.05 M EDTA (pH 9.0), 0.25 M sucrose, 4 mM β -mercaptoethanol]. Extracts were passed through two layers of cheesecloth and centrifuged at 1000 $\times$ g for 5 min. The supernatant was loaded onto step gradients in ultracentrifuge tubes. The step gradient consisted of a 0.5 ml of 1.5 M sucrose [in 40 mM Tris (pH 9.0), 100 mM KCl, 30 mM Mg acetate, 5 mM EGTA (pH 9.0), 8 mM β -mercaptoethanol] beneath 3 ml of 0.5 M sucrose. The polyribosomes were

pelleted through the gradient at 111,000 $\times$ g for 2 h 40 min. The gradient was carefully aspirated to preserve the pellet, and the RNA-protein pellets were resuspended [10 mM Tris (pH 8.0), 2 mM EDTA (pH 8.0), 1 M NaCl, 0.1 % SDS]. RNA was purified by phenol/chloroform/isoamylalcohol extractions and ethanol precipitation. Standard spectrophotometric methods were used for determining the concentration of the RNA. For sqRT-PCR, cDNAs were synthesized from 5 μ g of total RNA primed with primers specific for Gus (primer sequences shown in Table S1) using M-MuLV reverse transcriptase (MBI Fermentas, Lithuania).

Protein isolation and western analysis

Soluble proteins from vegetative tissues of *Arabidopsis* and rice were isolated in N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES) buffer [25 mM HEPES, pH 7.5, 0.5 % Triton X-100, 200 mM NaCl, 0.5 mM EDTA, 10 mM MgCl₂, and 1 $\times$ protease inhibitor cocktail (G-biosciences)]. Total seed proteins were isolated in Tris buffer (30 mM Tris-Cl, pH 8.5, 1 mM EDTA, 1 % SDS, and 1 $\times$ protease inhibitor cocktail). Proteins from rice pollen were isolated using HEPES extraction buffer. Protein samples were separated on 10 % SDS gel. For immunoblots, gels were processed by electroblotting on nitrocellulose membrane (Hybond C-super membrane; Amersham-Pharmacia, USA) as described by Katiyar-Agarwal et al. (2003). For analyzing the levels of OsClpB-C, anti-OsClpB-C (Pareek et al. 1995), and anti-At-ClpB-C, primary antibody (Agrisera, Sweden) and anti-rabbit secondary antibody (Sigma-Aldrich) with 1:5000 and 1:10000 dilutions, respectively, were used.

Results

Expression characteristics of 1-kb OsClpB-C promoter-driven Gus gene in transgenic *Arabidopsis*

The 1-kb OsClpB-C promoter sequence contains a functional HSE at position -272 to -279 from the translation initiation site (Fig. 1a). The in silico analysis indicated presence of two STRE sequences and an AP-1-binding element (elements responsive to heavy metal stress), an EBOXNAPA element (E-BOX; element conferring seed-specific expression), and an ABRE-like sequence in this 1-kb promoter (Fig. 1a). A canonical TATA-box-like element was lacking in the promoter. All the above sequences were marked taking “A” of ATG translation initiation codon as +1. A comparison of 1-kb OsClpB-C promoter with 1-kb promoters of *Zea mays*, *Arabidopsis thaliana*, and *Populus trichocarpa* ClpB-C genes revealed conservation of HSE, STRE, and EBOXNAPA elements (Fig. 1b). The PCR-amplified 1-kb OsClpB-C promoter fragment from PB1 rice genomic DNA template was

Fig. 1 In silico analysis of OsClpB-C gene promoter and its comparison with ClpB-C promoters of three different plant species. **a** Linear representation of 1-kb promoter region of OsClpB-C gene. ATG as well as selected regulatory elements is indicated. All elements are marked taking A of ATG as +1. **b** Comparative analysis for the cis-acting

elements present in 1-kb ClpB-C gene promoters of monocots *Oryza sativa* (OsClpB-C) and *Zea mays* (ZmClpB-C), dicot herb *Arabidopsis thaliana* (AtClpB-C), and a dicot tree species *Populus trichocarpa* (PtClpB-C). Different cis-elements are shown by different color codes

sequenced and cloned upstream to Gus reporter (construct 1kbOsClpB-Cpro:Gus; Fig. 2a). The 1kbOsClpB-Cpro:Gus construct was transformed in *Arabidopsis*, and stable, homozygous transgenic plants (1kbPro plants) were raised. Three homozygous transgenic lines, 1kbPro-1, 1kbPro-2, and 1kbPro-3, were analyzed.

Intense Gus activity was noted in 1kbPro seedlings upon HS (38 °C/2 h) treatment whereas Gus activity was not detected in control, unstressed seedlings (Fig. S1a). Similar observation was noted with leaves of 1-month-old 1kbPro plants (Fig. S1b). In MUG assays, 1kbPro seedlings showed remarkably high Gus activity following HS (38 °C/2 h) whereas control seedlings showed negligible activity (Fig. 2c). In sqRT-PCR assays with HS (38 °C/15 min; 38 °C/30 min)-treated 10-day-old seedlings, Gus transcript was HS-induced (Fig. 2b). Under heavy metal stress (20-μM solutions of arsenic, cadmium, copper, and cobalt salts; 6 h), Gus activity was lacking in 1kbPro seedlings. Gus activity was analyzed under control, unstressed conditions in inflorescence, pollen, young siliques and mature imbibed seeds of 1kbPro plants (Fig. S1c). Constitutive expression of Gus was

noted only in case of mature seeds among these stages. We have earlier noted that AtClpB-C expresses constitutively in developing *Arabidopsis* pollen (Mishra and Grover 2014). Anti AtClpB-C antibodies cross-react with OsClpB-C protein. Western analysis with anti AtClpB-C antibodies showed that OsClpB-C protein was below detectable levels in pollen of rice (Fig. 2f). Singla et al. (1998) showed that OsClpB-C protein accumulates in seeds at a high titer and disappears with progressing days of germination. The Gus protein and transcript were analyzed in 1kbPro seeds during the seed germination process. The MUG assays showed gradual decline in Gus activity with progressing stages of seed germination (Fig. 2d). Similarly, the level of Gus transcript as analyzed by sqRT-PCR was found to be highest in imbibed seeds (Fig. S2). The level of OsClpB-C protein was next analyzed by western analysis at various stages of development in rice seeds using anti OsClpB-C antibodies. The level of OsClpB-C was highest in imbibed seeds, which gradually declined (Fig. 2e). Contrastingly, AtClpB-C expression in *Arabidopsis* shows a significant increase during early seed germination (Mishra and Grover 2014). This reflects the probable

Fig. 2 Analysis of Gus activity in 1kbPro plants under heat stress (HS) and unstressed, control (C) conditions. **a** Schematic diagram of 1kbOsClpB-Cpro:Gus construct used to produce 1kbPro plants. **b** sqRT-PCR data showing Gus transcript levels under C and HS conditions. Lower panel shows the standardization with actin primers. **c** Quantitative estimation of Gus activity by MUG assays in the 1kbPro seedlings under C and HS. For each homozygous line, MUG assays were carried out in three replicates with standard errors shown by error bars. Significant Gus activity changes were determined statistically by a two-sample *t* test ($P < 0.05$) and indicated by asterisks. **d** MUG assays during

different stages of seed germination of 1kbPro-1 plants. MUG assays were carried out in triplicate, and standard errors are shown by error bars. **e** Western analysis showing the levels of OsClpB-C protein at various stages of rice (PB1) seed germination. Five micrograms of soluble proteins was analyzed. **f** Western showing the levels of OsClpB-C proteins in pollen. Control and heat-stressed rice (42 °C/2 h) protein samples from seedlings were used as controls. Two micrograms of total protein from seedling and 10 µg of total protein from pollen were analyzed

differences in the function of OsClpB-C and AtClpB-C in the respective species during early seed germination. However, this observation merits further work in future course.

Singh et al. (2012) have shown that 2-kb OsClpB-C promoter expresses Gus under heavy metal (As, Cd, Cu, Zn) stress as well as under HS in vegetative tissues and constitutively in rice seeds. Two-kilobase OsClpB-C promoter was next cloned upstream to Gus, and the construct was stably transformed in *Arabidopsis* (2kbPro plants). 2kbPro plants showed heat-induced expression of Gus in vegetative tissues. However, Gus activity was not detected under heavy metal stress even in 2kbPro plants (data

not shown). This suggests that the molecular factors governing ClpB-C gene regulation in response to heavy metal toxicity differ in rice and *Arabidopsis* plants. Further, like the case of 1kbPro plants, constitutive expression of Gus was noted in mature seeds of 2kbPro plants. The Gus activity was lacking in pollen of 2kbPro lines as well (Fig. S3).

Role of OsClpB-C 5'UTR in driving Gus expression

KOME database has two cDNA entries corresponding to OsClpB-C gene: (1) AK100903, cloned from flower tissue,

and (2) AK105433, cloned from cold-treated callus. The sizes of the 5'UTRs in AK100903 and AK105433 are 136 and 189 bases, respectively. To investigate the role of 5'UTR, 862-bp OsClpB-C promoter (lacking the 138 nt of the UTR from 1-kb promoter) was PCR amplified and cloned upstream to Gus (Δ UTRClpB-Cpro:Gus construct; Fig. S4). Δ UTRClpB-Cpro:Gus construct was transformed in *Arabidopsis* (Col-0), and stable, homozygous transgenic lines Δ UTR-1, Δ UTR-2, and Δ UTR-3 were obtained.

The 10-day-old Δ UTR seedlings were subjected to HS (38 °C/15 min; 38 °C/30 min), and Gus transcript expression was analyzed by sqRT-PCR. The Gus transcripts were induced in response to HS in the Δ UTR lines (Fig. 3a). The expression levels were largely comparable in 1kbPro and Δ UTR transgenics (Figs. 2b and 3a). The Gus transcripts attained highest expression within the first 30 min of HS; thereafter, the levels became stable and showed gradual decline in 1kbPro and Δ UTR lines (Fig. S5a, b). Next, 10-day-old HS (38 °C/2 h)-treated seedlings of Δ UTR and 1kbPro lines were analyzed for Gus protein expression (control seedlings of Δ UTR plants did not show Gus activity; Fig. S5c). Δ UTR lines showed poor Gus histochemical staining (Fig. 3b (ii)). In MUG assays, Gus activity was almost negligible in Δ UTR plants as compared to 1kbPro plants after HS (38 °C/2 h; Fig. 3b (iii)). In another set of experiment, 10-day-old Δ UTR and 1kbPro lines were subjected to 38 °C/15 min-22 °C/2-h treatment (i.e., 15-min HS followed by 2-h recovery at 22 °C). In this treatment, Δ UTR seedlings showed intense blue Gus stain (Fig. 3c (ii)) and Gus activity analyzed by MUG assays was almost comparable in Δ UTR and 1kbPro plants (Fig. 3c (iii)). Similar pattern of Gus activity in the two transgenic types was obtained upon 38 °C treatment for varying time points with and without 22 °C/2-h recovery treatment (Fig. S6). Like 1kbPro lines, Δ UTR lines showed high Gus activity in mature seeds under uninduced, control conditions (Fig. 4).

Analysis of 5'UTR for cryptic promoter activity

The 138-nt 5'UTR fragment was next analyzed for cryptic promoter activity. CaMV35S-GFP (pSM-GFP vector) and 5'UTR-GFP (in pSM-GFP background) constructs were employed in this experiment (Fig. 5a). First, the plasmid DNAs of CaMV35S-GFP and 5'UTR-GFP were bombarded in onion epidermal peels, in replicates of three each. Large numbers of cells with GFP signals were seen in the peels bombarded with the CaMV35S-GFP plasmid, but GFP expression was lacking in case of 5'UTR-GFP plasmids (Fig. 5b). To rule out any technical error for the lack of GFP fluorescence in the cells bombarded with 5'UTR-GFP plasmid, we next bombarded a physical mix of CaMV35S-GFP and 5'UTR-GFP plasmids. Onion peels bombarded with the

two DNAs together in physical mix recovered GFP signals in several cells (Fig. 5c).

Analysis of the association of Gus transcripts with polyribosomes

The 10-day-old 1kbPro-1 and Δ UTR-1 seedlings were analyzed for association of OsClpB-C 5'UTR with polyribosomes (Fig. 6a). These lines showed almost similar Gus transcript expression with total mRNA fractions. Polysomal RNA fraction was isolated from HS (38 °C/30 min) seedlings, and cDNA was synthesized. Prior to Gus transcript analysis, the amount of cDNA was normalized using actin-specific primers (Table S1). PCR conditions were similar as in case of PCR with cDNA prepared from total RNA (25 cycles). Requisite-sized amplicons were obtained for actin gene with cDNAs from Δ UTR-1 and 1kbPro-1 plants. However, amplicon corresponding to Gus was obtained in cDNA samples of heat-stressed 1kbPro-1 seedlings and not in Δ UTR-1 seedlings. PCR reactions using double amounts of cDNAs showed much higher Gus transcript in 1kbPro-1 as compared to Δ UTR-1 samples (Fig. 6b).

Analysis of 5'UTR region for its role as a translational effector

OsClpB-C 5'UTR was analyzed for translation enhancer activity. CaMV35S-GFP (referred to as CG) and CaMV35S-UTR-GFP (CUG) constructs were made, and stable transgenics were raised in PB1 rice. The presence of 138 bp 5'UTR-GFP transcript in CUG plants was confirmed through sqRT-PCR analysis (Fig. 7b). Two independent T₀ plants for each construct were analyzed. The 40-day-old CG and CUG T₀ plants were heat stressed (45 °C; 3, 5, and 7 h), and in each case, soluble protein from leaf tissues was analyzed for GFP expression. In western analysis, GFP protein levels in CUG plant showed a slight decrease in 3-h HS sample as compared to control. In the subsequent period, GFP levels showed an increase and the levels of GFP in 7-h stressed tissues were much higher than the 3-h stressed sample in CUG plant (Fig. 7c). On the other hand, GFP protein levels showed a continuous decline in CG plants with increasing durations of HS (Fig. 7e).

Discussion

While analyzing the transcriptional regulation of OsClpB-C gene in our previous study, we reported that OsClpB-C promoter expressed the reporter Gus gene in heat- and development-regulated manner in transgenic rice plants (Singh et al. 2012). It has earlier been seen that selective Hsp promoters can drive the reporter protein in a heat-

Fig. 3 Analysis of Gus transcript and protein in Δ UTR seedlings. **a** sqRT-PCR data for the levels of Gus transcript (*upper panel*) under control and HS (15 and 30 min) conditions. *Lower panel* shows the standardization with actin primers. PCR was performed for 25 cycles, and products were resolved on 1.2 % agarose gel. **b**, **c** Qualitative histochemical Gus staining and MUG assays in 10-day-old 1kbPro and Δ UTR seedlings after 38 °C/2 h (**b** (i)) and 38 °C/15'–22 °C/2 h (**c** (i)) HS

treatments. Histochemical staining (**b** (ii)) and MUG assays (**b** (iii)) in 1kbPro and Δ UTR seedlings under 38 °C/2-h treatment. Histochemical staining (**c** (ii)) and MUG assays (**c** (iii)) in 1kbPro and Δ UTR seedlings under 38 °C/15 min–22 °C/2-h treatment. MUG assays were performed in three replicates for each of the three homozygous lines of the two transgenics; standard errors are shown by *error bars*

Fig. 4 Qualitative histochemical Gus staining (**a**) and MUG assays (**b**) of 1kbPro and Δ UTR imbibed seeds. The MUG assays were carried out in three replicates for each transgenic line; standard errors are shown by error bars

regulated manner in trans-host species as well (Freeman et al. 2011; Khurana et al. 2013; Lee et al. 2007a; Saidi et al. 2005). This fact highlights that the heat shock response mechanism may be somewhat conserved in plants. We analyzed 1-kb ClpB-C promoters of *A. thaliana*, *O. sativa*, *Z. mays*, and *P. trichocarpa* and noted a significant conservation of HSE, STRE, and EBOXNAPA elements (Fig. 1b). To gain experimental support for the conservation of expression machineries in rice and *Arabidopsis*, we analyzed *Arabidopsis* transgenic (1kbPro lines) harboring 1-kb OsClpB-C promoter upstream to Gus. 1kbPro *Arabidopsis* seedlings expressed Gus transcript and protein in heat-inducible manner, suggesting that heat shock response machinery involved in ClpB-C expression is broadly conserved in rice and *Arabidopsis* plants.

5'UTRs have been suggested to play a critical role in the expression of Hsps during cellular stress. To examine the role of OsClpB-C 5'UTR sequence, transgenic *Arabidopsis* lines

Fig. 6 Analysis of the association of Gus transcripts with the polysomal fractions of 1kbPro-1 and Δ UTR-1 lines. **a** sqRT-PCR gel picture showing the levels of Gus transcript in the cDNA synthesized from total RNA of seedlings under control (C) and heat stress. **b** sqRT-PCR data showing the levels of Gus transcript in the cDNA synthesized from polysomal RNAs of 1kbPro-1 and Δ UTR-1 lines heat stressed at 38 °C/30 min. Lower panel shows the normalization with actin primers. In each case, PCR was performed for 25 cycles and products were resolved on 1.2 % agarose gel

Fig. 5 Analysis of 5'UTR for cryptic promoter activity. **a** Schematic representation of the constructs CaMV35S-GFP and 138 bp 5'UTR-GFP used in the analysis. **b** Confocal pictures showing the status of GFP signals in the peel bombarded with the respective constructs (shown by arrows). **c** Confocal pictures showing the GFP signals in peel bombarded with a physical mix of CaMV35S-GFP and 138 bp 5' UTR-GFP plasmids. GFP signals in peel bombarded with 138 bp 5' UTR-GFP are absent

Fig. 7 Analysis of translation enhancer activity in 138 bases of OsClpB-C 5'UTR sequence. **a** Schematic representation of the CaMV35S-UTR-GFP construct. **b** Confirmation of the status of transcripts in T₀ CUG (CaMV35S-UTR-GFP) plants. Forward primer corresponding to 138 bp UTR (Fp) and reverse primer corresponding to 250 bp of GFP transcripts (Rp) were used. PCR was performed for 25 cycles. **c** Western blot for the analysis of GFP protein expression in the CUG plants under indicated conditions. **d** Schematic representation of the CaMV35S-GFP (CG) construct. **e** Western blot for the expression of GFP protein in CG plants under similar conditions. Lower panel in each case represents the Coomassie-stained protein bands showing equal loading

harboring 862-bp OsClpB-C promoter (lacking 138 nt from 3' end of its 189 bp 5'UTR) upstream to Gus (Δ UTR lines) were analyzed. Δ UTR seedlings showed almost negligible levels of Gus protein after HS (38 °C/2 h; Fig. 3b). On the other hand, the levels of heat-induced (38 °C/15 min, 38 °C/30 min) Gus transcript were comparable in seedlings of 1kbPro and Δ UTR

lines (Figs. 2b and 3a). Considering that HS-inducible Gus transcript was formed in both the transgenic types, the difference in the Gus protein formation may be an outcome of post-transcriptional regulation. It was further corroborated by the absence of cryptic promoter activity in the 138-nt UTR fragment as accrued from the comparison of GFP expression in onion peels bombarded with CaMV35S-GFP and 5'UTR-GFP plasmids (Fig. 5b). The OsClpB-C 5'UTR thus seemingly has a role in the translation of the downstream open reading frame (ORF) during HS regimes. Importantly, we noted that Δ UTR seedlings formed Gus protein when these were placed for 2 h at optimal temperature of 22 °C post 38 °C/15-min HS (Fig. 3c). Yanguez et al. (2013) showed that only selective mRNAs involved in establishment of the HS response are translationally activated under HS conditions during which cap-dependent translation is severely impeded. This may suggest that translation through cap-dependent process is halted during HS and replaced by cap-independent process, in which the role of 5'UTR becomes critical. We propose that Gus transcripts formed in Δ UTR plants during the initial 15 min of HS were not translated into protein, as these may be lacking the ability for cap-independent translation. However, these

Fig. 8 Secondary structure of OsClpB-C 5'UTR as predicted by MFOLD program. Nucleotide sequence shows the LM1 enhancer (in underlined bold fonts at position -69 to -74) in the 138 bases of 5'UTR

transcripts were translated into Gus protein in recovery phase (22 °C/2 h) possibly with the resumption of cap-dependent translation process (Fig. 3c). It thus emerges that the HS-induced transcript of OsClpB-C chaperone is regulated post-transcriptionally in which the 138-nt region of the 5'UTR is a decisive factor.

Previous studies have shown that 5'UTR is important for loading of the ribosomes onto transcripts (Kawaguchi and Bailey-Serres 2005; Sherameti et al. 2002). We noted that the levels of Gus transcripts associated with the polysomal fraction were relatively higher in 1kbPro seedlings as against Δ UTR seedlings (Fig. 6b). It thus appears that the 5'UTR of OsClpB-C gene enabled higher levels of ribosomal loading to the transcripts during HS conditions. This may be a reason behind sustained and higher Gus expression in 1kbPro plants during HS. Further, 5'UTR has been implicated in translational enhancement in case of S-adenosylmethionine decarboxylase gene of *Brassica juncea* (Hu et al. 2005), RbcS1 of *Amaranthus hypochondriacus* (Patel et al. 2004), and Lat52 gene of tomato (Bate et al. 1996). 5' leader (also called Ω) of tobacco mosaic virus (TMV) has the ability to increase the translation by enhanced recruitment of eIF4F (Gallie 2002). Translational enhancement in case of tobacco photosystem I gene (psaDb) and *Arabidopsis* ferredoxin A gene (FedA) has been attributed to LM1 (TCTCAA) sequence present in the 5' UTR (Caspar and Quail 1993; Yamamoto et al. 1995). Through in silico analysis, we noted that 138 nt of OsClpB-C 5'UTR also contains LM1-like sequence (Fig. 8). Additionally, using MFOLD program (<http://mfold.rna.albany.edu/?q=mfold>), we noted that the 138-bp sequence of

OsClpB-C 5'UTR folds into a typical Y-shaped structure (with a free energy of $dG = -22.6$ kCal/mol; Fig. 8). This structure resembles with the shape of human Hsp70 5'UTR which reportedly contains IRES (Rubtsova et al. 2003; Vivinus et al. 2001). The maize ClpB-C 5'UTR is shown to harbor an IRES element (Dinkova et al. 2005). Thus, a combination of higher ribosomal loading, LM1-like enhancer activity, and IRES might result in increased Gus protein expression in 1kbPro seedlings during HS. Alternatively, the activity of LM1-like enhancer and/or IRES element might be the cause of higher ribosomal loading. The precise role of LM1-like enhancer through deletion analysis and presence of IRES element would be explored in subsequent studies.

Plant ClpB-C protein is expressed constitutively in seeds besides its HS-induced expression in vegetative tissues. Expression pattern of Gus protein in 1kbPro plants corroborated this attribute of ClpB-C expression. However, it was interesting to note that Δ UTR seeds accumulated Gus protein constitutively at levels comparable to 1kbPro seeds (Fig. 4). This indicates that the mechanism(s) governing the constitutive expression of ClpB-C in seeds is possibly different from the one deciding its HS-mediated expression in seedlings. The model presented in Fig. 9 depicts that the regulation of ClpB-C expression is different in seed and vegetative tissues: UTR appears redundant for constitutive expression of protein in seeds while it is obligatory in vegetative tissues where ClpB-C expresses upon HS. It may be the case that the heat-induced expression of ClpB-C protein needs 5'UTR for cap-independent expression while there is no such need of 5'UTR for constitutive expression. However, there is a distinct possibility that 5'UTR of ClpB-C transcript may not be needed for expression of the protein in seeds under optimal conditions, but its presence is possibly critical for seed germination during HS. Clearly, there is a need to understand the regulation of ClpB-C proteins in seeds in greater depth.

In summary, we show that OsClpB-C transcript is regulated post-transcriptionally in which 5'UTR plays a critical role. During HS, 5'UTR may provide advantage to the downstream ORF in terms of (a) maintaining uninterrupted and enhanced translation and (b) enabling translation through more ribosomal loading to maintain higher titer during persistent HS to meet the demanding stressful conditions. We infer that the high titer of ClpB-C protein achieved under HS conditions is a result of combinatorial activity involving both transcriptional and post-transcriptional regulation. We also note that 5'UTR is not required for the translation of OsClpB-C transcript in seeds under unstressed conditions.

Fig. 9 Model representing the differential mode of ClpB-C regulation in vegetative versus seed tissues. In vegetative tissues, ClpB-C transcript is essentially HS induced. Transcript is further regulated post-transcriptionally where 5'UTR-mediated cap-independent translation results in expression of ClpB-C protein. In seeds, ClpB-C is constitutive in expression and the role of 5'UTR appears redundant. ClpB-C transcript is possibly translated through cap-dependent manner

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