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ORIGINAL PAPER

The 5′ untranslated region of the *rbp1* mRNA is required for translation of its mRNA under low temperatures in the cyanobacterium *Synechococcus elongatus*

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Abstract The unicellular cyanobacterium *Synechococcus elongatus* has three RNA-binding protein (Rbp) genes, *rbp1, rbp2* and *rbp3*. The *rbp1* gene was upregulated by cold treatment while *rbp2* and *rbp3* expression decreased remarkably after exposure to cold temperatures. To investigate the mechanism underlying cold-induced *rbp1* expression, a series of *rbp1-luxAB* transcriptional fusion constructs were expressed in *S. elongatus* PCC 7942 under cold conditions. The results showed that the region from −33 to −3 of the transcription initiation site contains an essential sequence for basal transcription of the *rbp1* gene and that the 120-bp region (−34 to −153) does not contain critical *cis*-elements required for cold-shock induction. In contrast, mutational analysis carrying the 5′-untranslated region (UTR) of *rbp1-luxAB* translational fusions indicated that the 5′-UTR of *rbp1* plays an important role in cold induction of the *rbp1* gene product. Taken together, we conclude that the cold induction of *rbp1* may be regulated at a posttranscriptional level rather than at the transcriptional level.

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Introduction

Most organisms including bacteria respond to environmental changes and induce the expression and accumulation of a specific subset of proteins for adaptation (Barria et al. 2013; Graumann and Marahiel 1996; Thieringer et al. 1998). Likewise, cyanobacteria also respond to changes in environmental temperatures and adapt to temperature stress, such as cold stress. RNA-binding proteins (Rbps) were identified as cold-shock proteins in cyanobacteria 20 years ago (Sugita and Sugiura 1994; Sato 1995). They contain a single RNA recognition motif (RRM) (Kenan et al. 1991). In the unicellular cyanobacteria *Synechococcus elongatus* strains PCC 6301 and PCC 7942, two Rbps (Rbp1 and Rbp2) were shown to express differently in cold-treated cells (Mutsuda et al. 1999; Sugita et al. 1999). Rbp1 is barely detected at 30 °C while the production of Rbp1 is dramatically induced upon a downshift of temperature to 10 °C. In contrast, Rbp2 accumulates at similar levels during 25 h after this temperature downshift (Mutsuda et al. 1999). Similar cold-inducible expression of *rbp* genes was found in the multicellular cyanobacteria *Anabaena variabilis* M3 (Maruyama et al. 1999; Ehira et al. 2003), *Anabaena* sp. PCC 7120 (Ehira et al. 2003) and the bloom-forming cyanobacterium *Microcystis* sp. (Tan et al. 2011). Cyanobacterial Rbps are considered to be the counterparts of major cold-shock protein (Csp) in *Escherichia coli* or *Bacillus subtilis* (Yamanaka 1999; Graumann and Marahiel 1998).

We previously reported that *rbp1*-disrupted mutants of *S. elongatus* PCC 7942 grew very poorly at 20 °C, while the *rbp2* disrupted grew well at 20 °C (Sugita et al. 1999). Therefore, Rbp1 is considered to have an essential role in cellular function at cold temperatures. Based on nucleotide sequence alignment of the upstream region from the translation initiation codon of *rbp* genes of *A. variabilis* M3, putative *cis*-elements, RBS, Boxes I, II and III, for cold-inducible expression of *rbp* were proposed (Ehira et al. 2003, 2005). However, it is still unknown whether such sequence elements are responsible for cold-inducible expression of *rbp* genes.

In this paper, we identified a new member of the *rbp* genes, *rbp3*, by a similarity search in the complete genome sequence of *S. elongatus* PCC 6301 (Sugita et al. 2007). Such *rbp3* genes exist in cyanobacterial species whose genome sequences were completely determined. We carried out a comparative study to assess how three *rbp* genes, *rbp1*, *rbp2* and *rbp3*, are expressed under cold temperatures in *S. elongatus* PCC 6301. Moreover, we used *rbp1-luxAB* transcriptional fusions or translational fusions with deletions to identify the *cis*-acting sequence elements involved in the accumulation of *rbp1* transcript at cold temperatures. For this analysis, the transformable strain *S. elongatus* PCC 7942 was used. The *rbp* genes ant their upstream sequences are identical between *S. elongatus* strains PCC 6301 and PCC 7942 (Sugita et al. 1999).

Materials and methods

Culture conditions

Synechococcus elongatus PCC 6301 and PCC 7942 cells were grown at 30 °C in BG-11 medium (Sugita and Sugiura 1994) supplemented with 2 mM $NaHCO_3$ under constant light (15 μE $m^{-2}s^{-1}$) with shaking. For cold stress experiments, cells grown at 30 °C were adjusted to 20, 15 or 10 °C rapidly in an ice-water bath and were then cultured in the temperature-adjustable incubator. Cells grown under the different temperature conditions were collected and frozen in liquid nitrogen.

RNA extraction, northern and dot blot analysis

RNA was extracted by Isogen (Nippon gene, Japan) according to the manufacturer's protocol. The cells (20 mg) were lysed in 200 μl of hot Isogen (50 °C) and incubated at 50 °C for 10 min. The cell lysate was treated with chloroform, and RNA was precipitated with isopropanol. To purify RNA, the RNA preparation was treated with DNase I and was then treated with acid phenol and chloroform (1:1, v/v). For northern blot analysis, RNA (2 μg) was separated on a 1 % agarose gel containing formaldehyde and blotted on Hybond N^+ membranes (GE Healthcare Life Sciences). For dot blot analysis, RNA (1 μg) was spotted and fixed on a membrane. Hybridization probes for *rbp1*, *rbp2, rbp3*, *trnV*-UAC and 16S rRNA genes were amplified by the polymerase chain reaction (PCR) from the *S. elongatus* genomic DNA using gene-specific primers (Table S1). The probe for *luxAB* was amplified by PCR from pAM977 (Kondo et al. 1993) with luxAB-F and luxAB-R primers (Table S1). DNA probes were labeled with [α-^{32}P]dCTP, and northern or dot blot hybridization was carried out as previously described (Sugita and Sugiura 1994). Autoradiographic images were analyzed and quantified using a Fuji bio-imaging analyzer BAS-2000.

Construction of plasmids

Plasmid pALK9 was generated by insertion of a 2.1 kb promoter-less *Vibrio harveyi luxAB* gene (from the nucleotide position −125 with respect to the *luxA* initiation codon to +66 with respect to the *luxB* termination codon, derived from pAM977, Kondo et al. 1993) into the *Eco*RV and *Sal*I sites of cyanobacterial transformational vector pAM1573, which contained the *S. elongatus* PCC7942 chromosomal neutral sites (NS) II, a chloramphenicol resistance gene cartridge (*CmR*) and a *trpA* terminator (Andersson et al. 2000). To construct the transcriptional fusion plasmids P*rbp1::luxAB* and P*rbp2::luxAB*, the promoter regions of *rbp1* (nucleotide positions −153 to +127 relative to the transcription initiation site +1) and *rbp2* (−126 to +247) were amplified by PCR and were then inserted into the pALK9 at *Stu*I and *Xba*I sites.

To construct *rbp1* promoter deletion series, Prbp1, F93, F63, F33 and F3, the upstream regions of *rbp1* were amplified by PCR. Each PCR fragment was inserted into pALK9 at *Stu*I and *Xba*I sites. To construct a translational fusion plasmid Trbp1, the *rbp1* promoter region (−153 to +127) fused in-frame with the *luxAB* coding region (+16 from the initiation codon) was inserted into pAM1573. To construct *rbp1* 5′-UTR deletion series, TD1, TD2, TD3 and TD4, were generated by site directed mutagenesis of plasmid Trbp1 by PCR. All PCR primers are provided in Table S1.

Measurement of bioluminescence

To measure the luminescence from *Synechococcus* cells carrying *rbp1-luxAB* fusions, 100 μl of cell culture was transferred to a micro titer plate and mixed with 10 μl of a 0.05 % *n*-decanal (*n*-decyl aldehyde, Sigma) emulsion. Bioluminescence of the cell suspension was measured with a luminometer (ATTO Luminescencer JNR AB2100, Japan) immediately after the addition of *n*-decanal. The intensity of bioluminescence was expressed in counts of photons per minute per OD_{730}. Cell culture without the *luxAB* reporter was used to measure the background and subtracted from sample measurements.

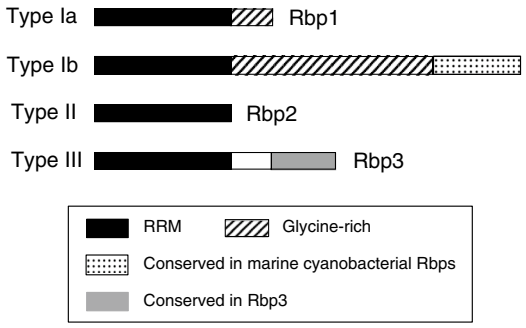

Fig. 1 Schematic structure of cyanobacterial Rbp proteins. The *filled*, *striped*, *dotted* and *gray regions* represent RNA recognition motif (RRM), glycine-rich domain, the conserved sequence 'SGWEDRSY' unique to marine cyanobacterial Rbps and the conserved sequence 'PDPRWA' found in Rbp3, respectively

Results and discussion

Cyanobacterial *rbp* gene families consist of three types of members

The complete genome sequence of *S. elongatus* PCC 6301 revealed an open reading frame (syc0747_d) homologous to *rbp1* and *rbp2* (Sugita et al. 2007). syc0747_d (142 residues) showed 41 and 40 % amino acid identity with Rbp1 (106 amino acids) and Rbp2 (99 amino acids), respectively, and was therefore designated as Rbp3 and the gene as *rbp3* (Fig. 1). The identical gene (Synpcc7942_0790) was found in *S. elongatus* PCC 7942 (http://www.genome.jp/kegg/catalog/org_list.html). Cyanobacterial Rbps can be classified into roughly three types I–III, based on the phylogenetic analysis of RRMs of Rbp proteins and the existence of C-terminal characteristic amino acid sequences (Fig. 1). Type I Rbps have a C-terminal glycine (Gly)-rich region. Type II proteins, including Rbp2, are small proteins without a C-terminal extension. Type III proteins have a non-Gly-rich C-terminal conserved sequence Pro-Asp-Pro-Arg-Trp-Ala 'PDPRWA' (Hamano et al. 2004). Type I proteins can be further divided into two subtypes Ia and Ib. Type Ia and Ib have a short (5–15 residues) and a long (30–80) Gly-rich region, respectively. Rbp1 belongs to Type Ia. Type 1b Rbps are distributed only among marine cyanobacterial species.

rbp1 transcript highly accumulated at cold temperatures while *rbp2* and *3* transcript levels largely decreased

To examine the effect of cold temperatures on the expression of the three *rbp* genes, *S. elongatus* PCC 6301 cells grown at 30 °C were transferred to various low temperatures and grown for 3 h. The *rbp1* transcript accumulated at substantial

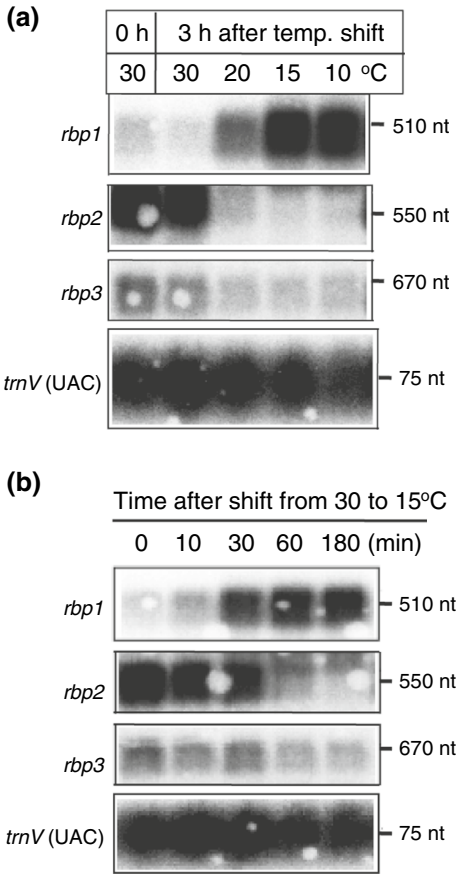

Fig. 2 Steady-state transcript levels of *rbp* genes after different temperature shifts. RNA (2 µg) was subjected to northern blot analysis using each *rbp*-specific probe. **a** *rbp* transcript levels 3 h after the indicated temperature shift. **b** Changes in the accumulation of *rbp* transcripts after a temperature shift from 30 to 15 °C relative to time (0, 10, 30 min, 1 and 3 h)

levels at 20 °C and at highest levels at lower temperatures (10 and 15 °C). In contrast, the *rbp2* and *rbp3* transcripts decreased remarkably after a temperature shift from 30 to 20 °C or lower (Fig. 2a). *rbp2* transcript (550 nt) is longer than *rbp1* (510 nt) because it has a longer 5′UTR. The transcript level of the $tRNA^{Val}$ gene (*trnV*-UAC) located upstream of the *rbp1* gene was not affected by low temperature shifts.

Next, we investigated the kinetics of changes in *rbp* transcript levels after this temperature shift. The *rbp1* transcript level rapidly increased within 30 min and peaked at 1 h after the temperature shift from 30 to 15 °C (Fig. 2b). By contrast, the *rbp2* transcript level largely decreased after 60 min following a downshift in temperature and appeared at a negligible level after 3 h. The level of *rbp3* transcript also declined after 60 min after a temperature downshift but was maintained at half the level of the control (i.e., before cold shock) (Fig. 2b). This suggests that *rbp3* may play a certain role in the cellular function at cold temperatures.

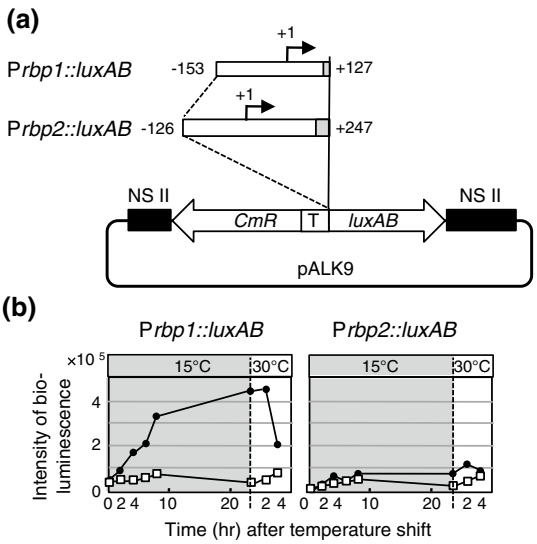

Fig. 3 Detection of cold-shock expression of *rbp* genes by a *luxAB* reporter assay. **a** A reporter vector pALK9, which contained the *luxAB* gene, the chloramphenicol resistance gene (*CmR*), *trpA* terminator (T) and genome neutral site (NSII) for homologous recombination, was used throughout the present study. Each promoter region of the *rbp1* or *rbp2* genes was inserted into pALK9. **b** Bioluminescence from the reporter cells was measured at 0, 2, 4, 6, 8, 24 h after they were transferred from 30 to 15 °C (*closed circles*). *Dashed lines* indicate the point of temperature shift from 15 to 30 °C, and bioluminescence was measured at 2 and 4 h after this temperature shift. Control luminescence of each reporter cell under constant temperature (30 °C) is indicated as *open squares*

The conspicuous changes to *rbp* transcript level after exposure to low temperatures can be due to changes in either transcriptional activity or mRNA stability. To investigate this possibility, the region upstream from each *rbp* coding region was fused to the *luxAB* genef and was integrated into the PCC 7942 genome neutral site NSII (Fig. 3a). The temperature shift from 30 to 15 °C induced bioluminescence in *rbp1-luxAB* and the intensity increased tenfold (Fig. 3b, left panel). The intensity of bioluminescence decreased rapidly after a temperature upshift from 15 to 30 °C. Such a cold response was not observed for *rbp2-luxAB* (Fig. 3b). This indicates that the *luxAB* can be used as a reporter assay for investigating the regulatory mechanism of *rbp1* gene expression under cold temperatures.

Involvement of the upstream region from the transcription initiation site of *rbp1* in cold-shock induction

To address how the accumulation of *rbp1* transcript is regulated under cold temperatures, we carried out in vivo reporter assays of *S. elongatus* PCC 7942 cells carrying a series of deletion mutants of the *rbp1* promoter region. The cells were grown at 30 °C and then transferred to 15 °C for 24 h. Bioluminescence from these cells was then measured. The bioluminescence intensity for P*rbp1::luxAB* containing the −153 to +127 region peaked at 15 °C and showed a 4.5-fold increase at 15 °C relative to 30 °C (Fig. 4a). As a control, P*rbp2::luxAB* did not exhibit any increase in bioluminescence or *luxAB* transcript levels. Prbp1 carrying the region from −153 to +97 gave 30 % lower intensity than P*rbp1::luxAB* at both temperatures 30 and 15 °C. This suggests that the +97 to +127 region containing the first 15-bp *rbp1* coding region is probably required for high expression of *rbp1* (Fig. 4a, c, d). Bioluminescence levels at 15 °C gradually decreased after further deletion of the upstream region from the transcription initiation site (F93, F63 and F33) but were maintained at about 5–8-fold higher level than at 30 °C. The *luxAB* transcript levels were 1.5- to 3-fold higher at 15 °C than at 30 °C in all the transformed cells (Fig. 4b, right panel). In contrast, F3 exhibited a background level of bioluminescence intensity even at 30 or 15 °C. Negligible levels of *luxAB* transcripts also accumulated at both temperatures (Fig. 4b). These results suggest that the region from −33 to −3 may contain an essential sequence for basal transcription of the *rbp1* gene and that the 120-bp region (−34 to −153) from the transcription initiation site of *rbp1* does not contain critical *cis*-elements required for cold-shock induction (Fig. 4c). However, we cannot exclude the possibility that the region from −33 to −3 is involved in not only basal transcription but also cold-inducible transcription of the *rbp1* gene.

Rbp1 mRNA is stabilized at low temperatures

The stability of *cspA* mRNA is known to play an important role in the cold-shock activation of *cspA* (Fan et al. 1997). To assess the possibility of posttranscriptional regulation of *rbp1* expression, we tested *rbp1* mRNA stability in cells treated by an inhibitor of RNA polymerase, rifampicin. Rifampicin (50 μg ml^{-1}) was added to the cold (15 °C)-treated wild-type PCC 7942 cells, immediately after the temperature upshift, and then RNA was extracted at different time points. As shown in Fig. 5, *rbp1* mRNA was extremely unstable at 30 °C and more than 95 % of the *rbp1* mRNA was rapidly degraded within 10 min after the temperature shift from 15 to 30 °C, whereas *rbp1* mRNA was not degraded at 15 °C. This result indicates that *rbp1* mRNA stability is markedly altered by different growth temperatures.

Role of *rbp1* 5′-UTR in cold-shock induction

The upstream sequences that are conserved among the cold-regulated *rbp* genes in *Anabaena* PCC 7120 and *A. variabilis* M3 were suggested to be involved in the regulation of cold-inducible expression (Maruyama et al. 1999;

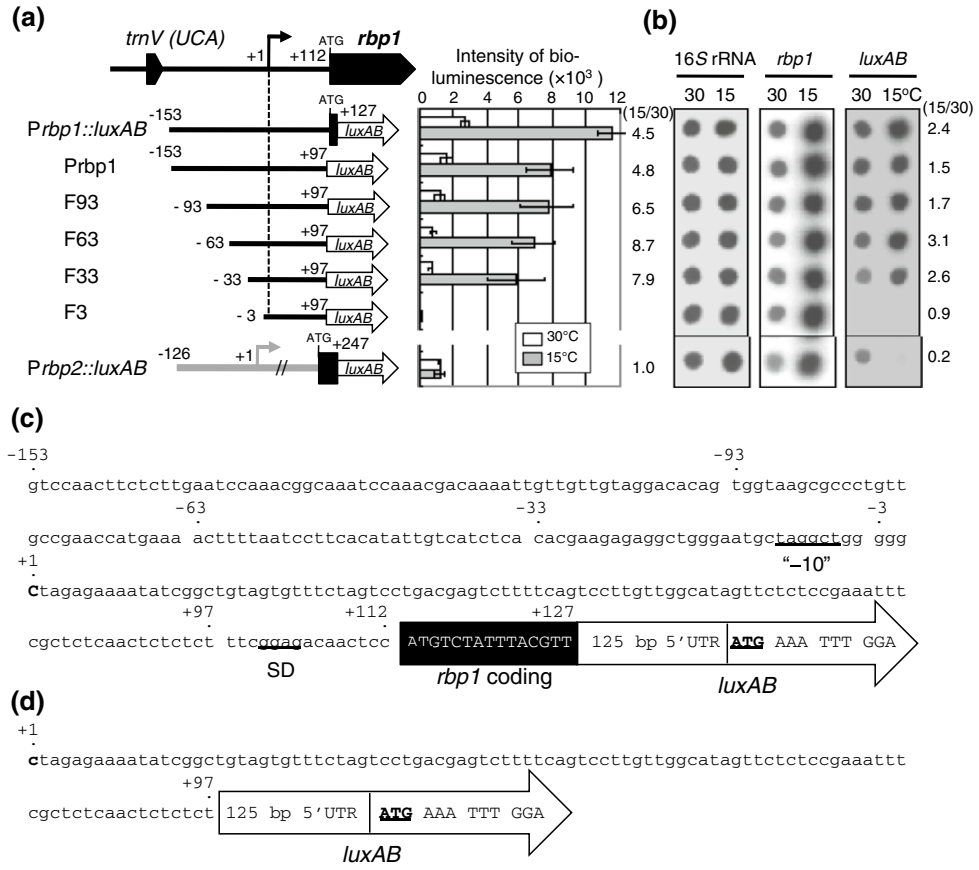

Fig. 4 Deletion analysis of the upstream sequence of the transcription initiation site of the *rbp1* gene. **a** *rbp1-luxAB* transcriptional fusions with various truncated upstream sequences of the transcription initiation site were constructed. Each construct was introduced into *S. elongatus* PCC 7942 cells. Bioluminescence was measured 24 h after a temperature shift from 30 to 15 °C. The data represent an average of five independent experiments. The relative level at 30 to 15 °C for each construct is shown on the *right* of the *graph*. **b** Steady-state levels of 16S rRNA, *rbp1* and *luxAB* transcripts at 0 or 3 h after the temperature downshift were analyzed by dot blot hybridization. **c** The nucleotide sequence of the P*rbp1::luxAB* construct is shown. Nucleotide numbers are indicated and that of the transcription initiation site, represented by a *bold capital* '**C**,' is +1. Capital letters start from ATG of the *rbp1* coding region. Two *underlines* represent a putative −10 sequence and a Shine–Dalgarno (SD)-like sequence, respectively. **d** The nucleotide sequence downstream from the transcription initiation site of Prbp1, F93, F63, F33 and F3 construct is indicated. The upstream sequence of each construct is not shown

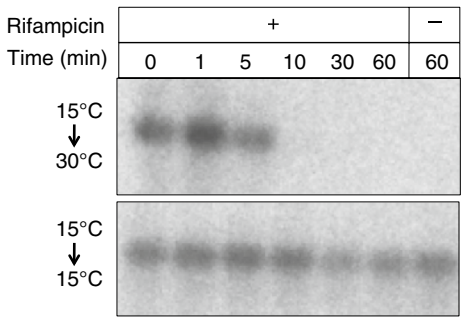

Fig. 5 Stability of *rbp1* mRNA after temperature shift. Cultures were incubated at 15 °C and then shifted to 30 °C or maintained at 15 °C after the addition (+) or no addition (–) of rifampicin (50 μg ml^{-1}). RNA was then extracted at different time points as indicated, and northern blot hybridization was carried out as described in "Materials and methods" section

Ehira et al. 2003, 2005). Similar conserved sequences, Boxes I, II and III, are also present in the 5′-UTR of the *S. elongatus rbp1* gene. To investigate the role of these sequences in *rbp1* expression under cold temperatures, we performed in vivo reporter assays of *S. elongatus* PCC 7942 cells carrying a series of a 25-bp deletion within the *rbp1* 5′-UTR (Fig. 6a, b). Each deletion construct carries the *rbp1* translated region (15 bp) fused in-frame to the translated region of *luxAB* (Fig. 6b, c). Trbp1 cells exhibited over 70-fold higher bioluminescence at 15 °C than at 30 °C. To our surprise, TD2 cells only emitted high bioluminescence (over 30-fold higher level at 15 °C than at 30 °C) while TD1, TD3 and TD4 exhibited very low bioluminescence. Besides, *luxAB* mRNA was detected at a low level in TD1, TD3 and TD4 cells both at 30 and 15 °C (Fig. 6b, right panel). This suggests that *rbp1-luxAB*

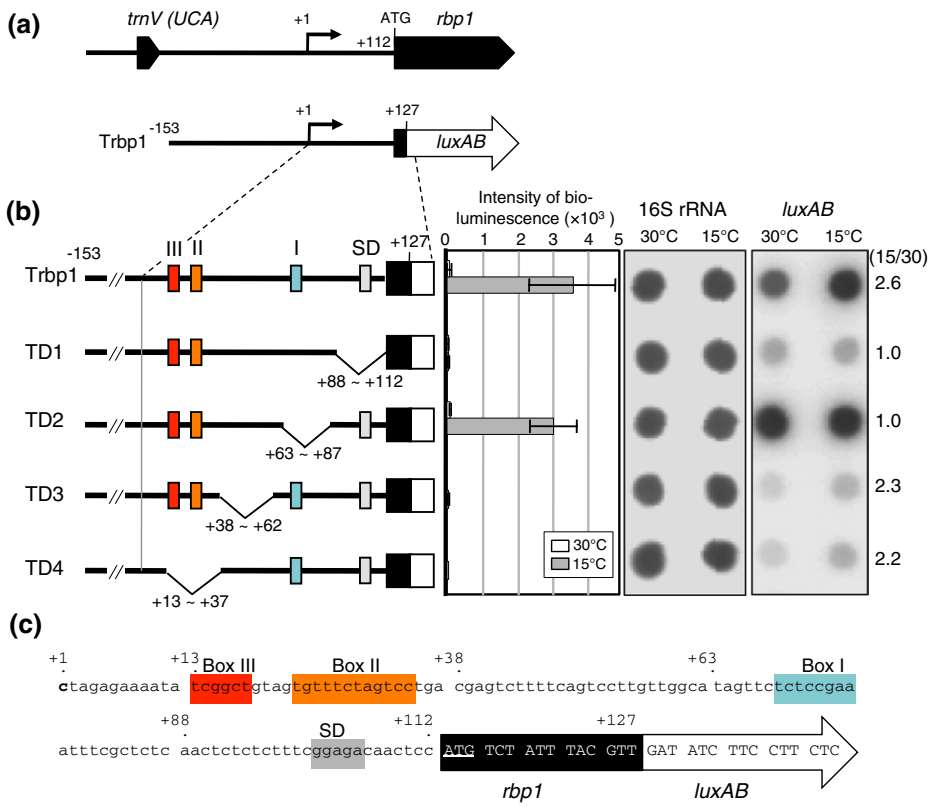

Fig. 6 Deletion analysis of the 5′-UTR of *rbp1* transcript, **a** The region from −153 to +127 of the *rbp1* gene was translationally fused to the *luxAB* coding sequence, which was inserted into pAM1573 to generate Trbp1. **b** For deletion analysis of the 5′-UTR, a 25 bp each in the 5′-UTR of Trbp1 was deleted to construct TD1 to TD4. SD-like, Box I, Box II and Box III, are presented as different *colored boxes*, respectively. Each construct was introduced into *S. elongatus* PCC 7942 cells and bioluminescence was measured as described in Fig. 4. Steady-state levels of the 16S rRNA and *luxAB* transcript at 0 or 3 h after the temperature downshift were analyzed by dot blot hybridization. **c** The nucleotide sequence of the Trbp1 construct is shown. SD-like, Box I, Box II and Box III, are presented as different *colored boxes*, respectively (color figure online)

mRNAs lacking SD-like sequence or Boxes II and III may be unstable in TD1, TD3 and TD4 cells. Further experiments, however, are needed to demonstrate how 5′-UTR is involved in stabilization of *rbp1-luxAB* mRNA. In contrast, *rbp1-luxAB* mRNA accumulated at a threefold higher level in TD2 cells than Trbp1 cells at 30 °C. This suggests that the region (from +63 to +87) containing Box I may be involved in destabilizing *rbp1* mRNA at 30 °C. In TD2 cells, the bioluminescence levels significantly increased at 15 °C although the *rbp1* mRNA levels were not largely changed at both at 30 and 15 °C under our reporter assay conditions. This suggests that the 5′-UTR of *rbp1* mRNA controls the translation.

In *A. variabilis* M3, an *rbpAI* transcript that lacks the entire 5′-UTR did not accumulate both at high (38 °C) and at low (22 °C) temperatures (Ehira et al. 2005). Taken together with our result and that of others (Ehira et al. 2005), we speculate that *rbp1* mRNA stabilization at 15 °C requires the regions containing the SD-like sequence, Boxes II and III. In 5′-UTR of *rbp1* mRNA, a stable secondary structure (minimum free energy −19.5 kcal/mol) could be formed at 30 °C and could hide the SD sequence preventing the entry of ribosomes, as shown in Fig. 7. The 5′ half of the 5′-UTR containing Boxes II and III might be involved in the formation of a stable secondary structure. Alteration of the secondary structure at different temperatures might require some unknown factor(s).

E. coli and *B. subtilis* Csp proteins are considered to play a role in RNA stability at cold temperatures (Goldenberg et al. 1996; Fan et al. 1997; Giuliodori et al. 2010). Regulation of the *cspA* gene of *E. coli* has been extensively studied and posttranscriptional mechanisms play a major role in cold adaptation (Goldenberg et al. 1996; Brandi et al. 1996; Gualerzi et al. 2003). Moreover, the *cspA* mRNA itself plays a role as a thermosensor to modulate translation of the cold-shock protein CspA (Giuliodori et al. 2010). In the context of posttranscriptional regulation by changing mRNA stability, the expression of *S. elongatus* PCC 7942 *rbp1*, *A. variabilis* M3 *rbpAI* and *E. coli cspA*

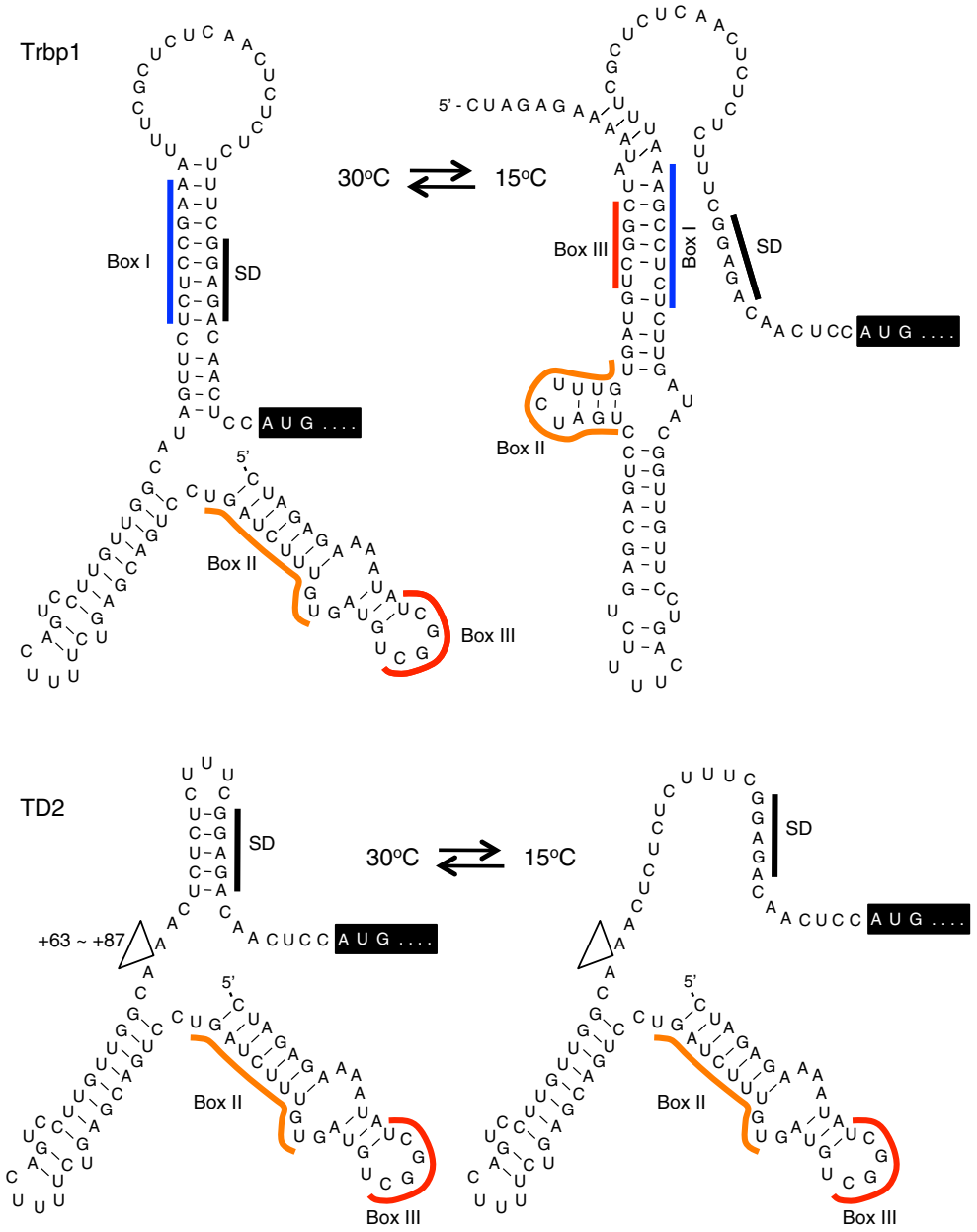

Fig. 7 Possible secondary structures of the 5′-UTR of *rbp1* mRNA. Secondary structures of the *rbp1* transcript at different temperatures were predicted using MFOLD Web Server (http://unafold.rna.albany.edu/?q=mfold/rna-folding-form). SD-like, Box I, Box II and Box III are indicated by different *colored thick lines*. The *outlined* ATG indicates the translation start codon of the *rbp1* coding region. The *triangle* represents the position of a 25-nt deletion (+63 to +87) (color figure online)

genes show a similar pattern in response to cold stress but the detailed mechanism, including *cis*-elements and *trans*-acting factors, involved in this cold regulation might differ RNA structural rearrangement of *rbp1* mRNA exposed to cold temperatures may require other cold shock-induced protein(s), such as RNA helicase (Chamot et al. 1999; Rosana et al. 2012). This possibility remains to be further addressed.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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