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The bean *PvSR2* gene produces two transcripts by alternative promoter usage

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Abstract

The bean (*Phaseolus vulgaris*) stress-related gene number 2 (*PvSR2*) is heavy metal-inducible. Here, the intron of *PvSR2* (I-PvSR) within the coding sequence was isolated and characterized. I-PvSR exhibited a weak and constitutive promoter activity and enhanced the *PvSR2* promoter activity in transiently transformed tobacco protoplasts. The transcription start site of I-PvSR promoter was mapped 72 bp upstream of the 3′-splice site. The shorter *PvSR2* transcript (768 nt) in bean is generated from this intronic promoter and lacks the last 56 bases of 3′-end sequence of longer *PvSR2* transcript (829 nt) by utilizing an alternative polyadenylation site. Quantitative competitive PCR analysis further revealed that two transcripts were differently accumulated in response to Hg^{2+}-exposure and the longer transcript was more abundant than the shorter one. These results demonstrate an additional non-metal inducible transcription of *PvSR2* via alternative intronic promoter usage and provide new insights into expression mechanism of metal inducible gene.

Keywords: Alternative polyadenylation site; Heavy metal; Intron; Intronic promoter

Metal responsive gene transcription in eukaryotic organisms has been well documented and the metallothionein (MT) gene is the most intensively studied and best understood example of metal-regulated transcription units [1]. Introns and the act of their removal by the spliceosome can regulate gene expression at many levels [2]. However, the intron-regulated transcription of heavy metal-inducible gene is largely unknown. The metal-induced expression of sea urchin *SpMTA* gene is enhanced by its first intron [3]. Cadmium stress but not other stress conditions retains the intron of maize *Bz2* gene to produce unprocessed *Bz2* transcript possibly functioning as detoxification agent of heavy metal [4]. Such knowledge can help to understand the molecular mechanism of heavy metal-regulated gene expression in more detail.

PvSR2 (*Phaseolus vulgaris* Stress-Related gene No. 2) (GenBank Accession Nos. U54704 and DQ109992) isolated from bean was proven to be a heavy-metal specific responsive gene previously in our laboratory, and its promoter is heavy metal inducible [5,6]. In this work, we showed that the *PvSR2* gene is also transcribed from a second weak promoter located within its single intron, generating a less-abundant, non-metal inducible shorter transcript with an alternative polyadenylation site.

Materials and methods

Plant materials and stress treatment. Tobacco plants (*Nicotiana tabacum* L. cv. W38) were grown in Murashige-Skoog (MS) medium for three weeks as described previously [6]. $HgCl_2$-stress treatment of bean (*Phaseolus vulgaris* L. cv. Saxa) was performed as described previously [6].

Construction of recombinant plasmids. The intron-containing and intronless *PvSR2* promoter fragments were amplified by PCR using the common promoter forward primer (PFP, 5′-CCCAAGCTTTGCA GACATCGTTTTGTATT-3′) and either exon 2 reverse primer (ERP2,

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5′-GCTCTAGATTGCTCTTCTTTTCTCTCATC-3′) or exon 1 reverse primer (ERP1, 5′-GCTCTAGATTGCTCTTCTTTTCTCTCATC GATTTGTTGGGGTTGGGGTTC-3′), respectively. The intron fragment flanking part exon sequences was also obtained by PCR with a pair of primers: ERP2 and exon 1 forward primer (EFP1, 5′-CCCAAGCTT GAACCCCAACCCCAACAAATC-3′). The underlined sequences of all above primers indicate the *Hin*dIII and *Xba*I restriction endonuclease sequences. The PCR products were introduced to *Hin*dIII/*Xba*I sites of pBI221 (Clontech) instead of the cauliflower mosaic virus (CaMV) 35S promoter fragment to yield the construct termed pBinMRP, pBMRP, and pBIN, respectively. The promoter or intron region of constructs was confirmed by sequencing. A *Hin*dIII/*Eco*RI fragment containing the intron–*GUS* cassette from pBIN was inserted into the *Hin*dIII/*Eco*RI sites of pBI121 (Clontech) to generate construct pBIN–GUS for the transformation of tobacco.

Transient expression assay. Transient transformation of tobacco mesophyll protoplasts with 20 μg of a test construct and 5 μg of a *CaMV 35S:luciferase* (*Luc*) control vector was performed as described previously [6]. The protoplasts transfected with either construct were then divided into two aliquots, one re-suspended in 2 mL heavy metal-free liquid MS medium [6] and the other in the same medium containing 25 μM $HgCl_2$. The samples were incubated in the dark at 25 °C in 3-cm diameter Petri dishes and transient gene expression was assayed at 24 h post-transformation. Extraction of GUS and LUC from transformed protoplasts and their assay were performed as described before [6]. GUS activity was normalized against LUC activity and expressed as a ratio of GUS/LUC units. The data are presented as means ± SD from three independent experiments.

Plant transformation. *Agrobacterium tumefaciens*-mediated tobacco transformation was performed according to published procedures [7]. GUS staining assay of transiently transfected tobacco leaf discs was performed at 3 days post-transformation as described by Jefferson [8]. The transgenic tobacco discs were grown in MS agar medium supplemented with 100 mg/L kanamycin and 0.5 mg/L α-naphthaleneacetic acid (NAA) for three weeks, and the kanamycin-resistant green calluses were selected for further analysis.

5′ Rapid amplification of cDNA ends (RACE). Total RNA was isolated from the kanamycin-resistant green calluses of transgenic tobacco using a Trizol solution (Gibco-BRL). 5′-RACE was carried out using a SMART RACE cDNA Amplification kit (Clontech) on adapter-ligated cDNA, synthesized from 2 μg of total RNA. The PCR was performed using a *GUS*-specific reverse primer (GusRP, 5′-ATCCAGACTGA ATGCCCACAGG-3′) by touchdown PCR program as described by the manufacturer. The PCR fragments were cloned into a pMD18-T vector (TaKaRa) and sequenced. Four separated positive clones were sequenced for mapping the transcription start site accurately.

RT-PCR and PCR analysis. To obtain the full-length longer *PvSR2* cDNA or shorter one, template RNA for RT-PCR was extracted from the leaves of bean with $HgCl_2$ stress (+) for 6 h or without heavy metal treatment (−) using a Trizol solution (Gibco-BRL), followed by RNase-free DNase (TaKaRa) treatment. RT-PCR was carried out using a common reverse transcription primer oligo$(dT)_{18}$ and either longer *PvSR2* forward primer 1 (LFP1, 5′-AATTTCCACCAAAACCTAAACCC-3′) corresponding to the 5′-end sequence of longer *PvSR2* cDNA or TSSP-down primer specific for the 5′-end sequence of shorter *PvSR2* cDNA according to the instructions of TaKaRa RNA LA PCR Kit, respectively. The genomic DNA fragment of *PvSR2* was amplified by PCR using bean genomic DNA as a template and two primers: LFP1 and the longer *PvSR2*-specific reverse primer 1 (LRP1, 5′-AAAGTAACAAA ACTGCCACA-3′) specific to the 3′-end sequence of longer *PvSR2* cDNA. The PCR products were cloned into a pMD18-T vector and sequenced to yield pUC-LPvSR2, pUC-SPvSR2, and pUC-gPvSR2, respectively. Four separated positive clones of pUC-LPvSR2 or pUC-SPvSR2 were sequenced for mapping 3′-end sequence accurately.

Quantitative competitive PCR (QC-PCR). The QC-PCR was employed to assess the level of two *PvSR2* transcripts' expression. The synthesis of native and competitor DNA templates as internal standards for the longer or shorter cDNA was carried out by PCR amplification according to the method described previously [9]. Briefly, the primary PCR was performed on pUC-LPvSR2 or pUC-SPvSR2 template using either the longer *PvSR2* forward primer 2 (LFP2, 5′-ACA ATCTTCACAGTTCCATC-3′) or the shorter transcript forward primer 2 (SFP2, 5′-TTTTAAAGATGAGAAGATTCC-3′) and the common longer *PvSR2*-specific reverse primer 2 (LRP2, 5′-GATCGGCGTATTTG AGGAGC-3′), respectively. The nested PCR amplification was carried out with either LFP2 or SFP2 and the common nested longer *PvSR2*-specific reverse primer 2 (NLRP2, 5′-GATCGGCGTATTTGAGGA GCGTCTTCACGGCGTCCTCCAC-3′), respectively. After nested PCR amplification, it was gel-purified and quantified by spectrophotometry.

Total RNA was extracted using a Trizol solution (Gibco-BRL) from the leaves of bean without (−Hg) or with $HgCl_2$ stress (+Hg) for 6 h followed by RNase-free DNase (TaKaRa) treatment, respectively. Total RNA (6 μg) was reverse-transcribed according to the manufacturer's protocol of AMV first strand cDNA synthesis kit (BBI) using oligo$(dT)_{18}$ as a primer at 42 °C for 1 h in a total reaction volume of 40 μL. Co-amplification of a constant amount of shorter cDNA (derived from 300 ng of RNA[−Hg] or RNA[+Hg]) or longer cDNA (derived from 100 ng of RNA[−Hg] or 25 ng of RNA[+Hg]) with serial 1:2 dilutions of its mimic was performed. PCR was negative when the reaction was performed without prior RT reaction. PCRs were performed for 35 cycles at annealing temperature 52 °C using primers SFP2/LRP2 for the shorter transcript or for 30 cycles at annealing temperature 55 °C using primers LFP2/LRP2 for the longer transcript. The 5 μL PCR products were separated on agarose gel stained with ethidium bromide, and the intensity of the bands was quantified by Adobe PhotoShop version 6.0 software. The amount of specific cDNA of interest was determined from the competition curve derived by plotting the ratio of the densities of the target to mimic PCR products co-amplified in the same tube vs. the concentration of the mimic. The level of transcript was expressed as the "number of copies/μg of total RNA". Data were means ± SD of three independent PCR replicates from each RT-reaction sample.

Results

A single intron (I-PvSR) locates inside the coding region of PvSR2

The full-length *PvSR2* cDNA was obtained by RT-PCR from RNA of Hg^{2+}-treated bean leaves (data not shown). DNA sequencing of four independent RT-PCR products confirmed the previously unidentified 20-bp sequences between the putative poly(A) recognition sequence (AATAAA, underlined in Fig. 1) and the poly(A) tail. The full-length *PvSR2* cDNA (829 bp) contained an entire open-reading frame (ORF) which encoded a protein of 200 amino acids (Fig. 1). The genomic DNA of *PvSR2* gene was further amplified from the genomic DNA of bean by PCR with a pair of primers corresponding to both 5′ and 3′-end sequences of *PvSR2* cDNA.

Alignment of genomic DNA with cDNA sequence of *PvSR2* revealed a 444-bp long intron (GenBank Accession No. DQ109993). It located at 30 bp downstream of the ATG start codon of *PvSR2*. The intron of *PvSR2* (I-PvSR) had a high AU content (71.5%) and was bordered by the 5′-GT and AG-3′ consensus splicing sequence of introns (Fig. 1).

I-PvSR enhances the PvSR2 promoter activity in vivo

A set of chimeric expression vectors were constructed using β-glucuronidase (GUS) gene as a reporter to evaluate

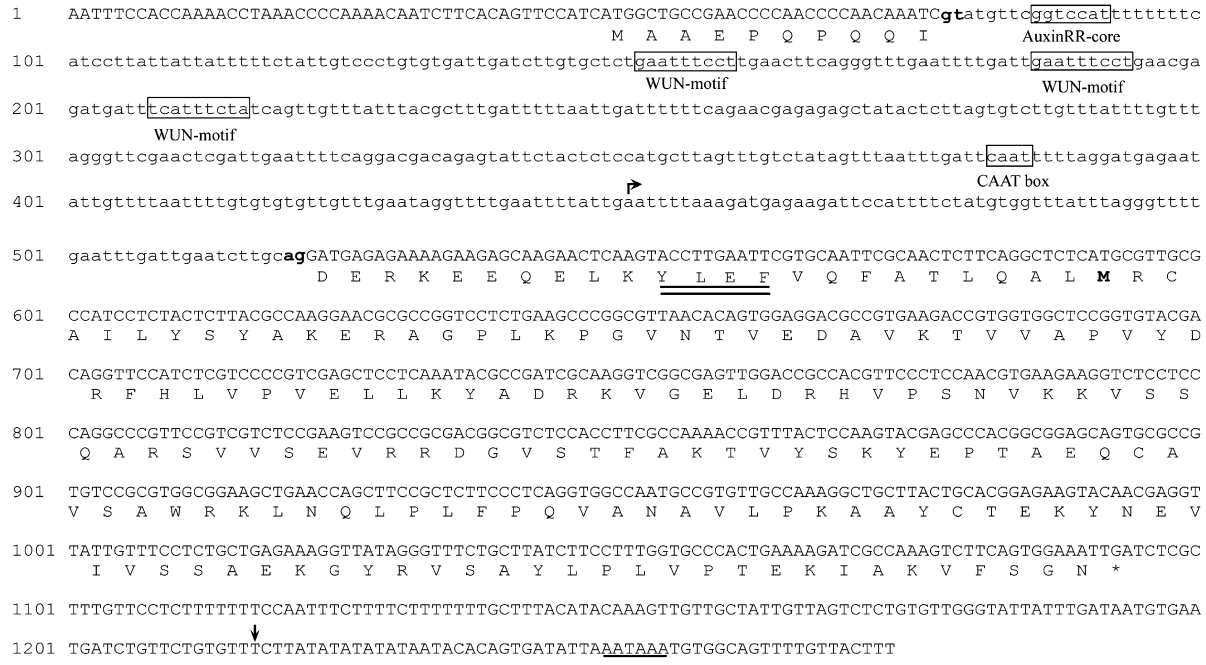

Fig. 1. The genomic sequence of *PvSR2* gene. The coding sequence is shown in upper case while the intron, as determined by comparison to the cDNA clone, is shown in lower case. 5′ or 3′ exon/intron consensus splicing sequence is marked in bold. The 5′-end of shorter *PvSR2* transcript, as determined by four identical 5′-RACE products, is indicated in a bent arrow, while its poly(A) site, as confirmed by four identical RT-PCR products, is shown in a vertical arrow. The conserved methionine codon, which may be translation initiation site for shorter transcript, is in bold. The putative poly(A) recognition sequence is underlined. Double underlined sequences represent the putative tyrosine-based sorting signal for PvSR2 protein. The putative *cis*-elements present inside the intronic promoter sequence are boxed. AuxinRR, auxin-response region; WUN, wound-responsive element.

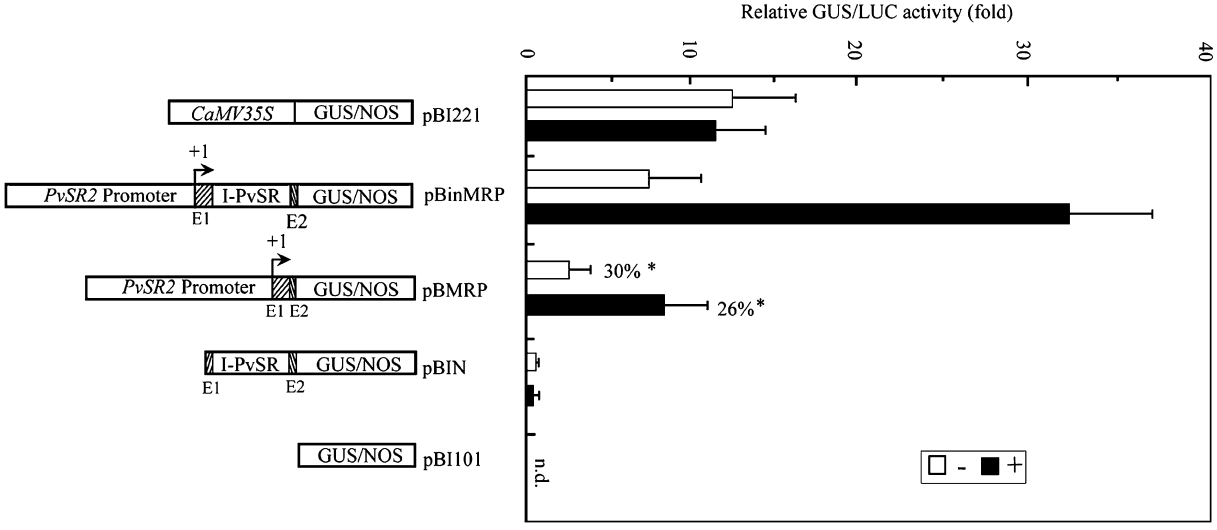

Fig. 2. Analysis the effect of I-PvSR on the *PvSR2* promoter activity *in vivo*. Schematic diagrams of the constructs used for transient expression assays are shown at left. The transcription start site was shown as +1. Exon 1 and 2 sequence are represented by E1 and E2, respectively. In each experiment, protoplasts transformed with each of the plasmids shown and a *CaMV 35S:Luc* construct (pBI221-Luc) were incubated for 24 h in heavy metal-free MS medium either with (+) or without (−) 25 μM $HgCl_2$. The plasmid containing a promoter-less *GUS* (pBI101) was used as a negative control, while the *CaMV 35 S:GUS* containing plasmid (pBI221) was used as a positive control. Activity, given as relative GUS/LUC units (fold), is averaged from three independent experiments. Bars indicate SD. Numbers above the bar of pBMRP sample indicate the percentage of GUS activity relative to the pBinMRP sample under condition of −Hg and +Hg, respectively, and its significant difference test was assessed by one-sided paired *t*-test ($^*P < 0.01$). n.d., not detectable.

the effect of I-PvSR on the *PvSR2* promoter activity in transiently transformed tobacco protoplasts in the presence (filled bars) and absence (open bars) of 25 μM $HgCl_2$ (Fig. 2) The *GUS* was preceded by the prolonged promoter region containing I-PvSR (pBinMRP), the same sequence without I-PvSR (pBMRP), or I-PvSR alone (pBIN). A *CaMV 35 S:Luc* construct (pBI221-Luc) was used as an internal control by co-transformation to normalize the

transformation efficiency in each experiment. The negative control (pBI101), which contains a promoterless *GUS*, had undetectable levels of GUS activity in this assay. The constitutive CaMV 35S promoter in pBI221 construct (positive control) showed no significant difference either in the presence or absence of $HgCl_2$ stress. However, GUS activities driven by the prolonged intron-containing or intron-less *PvSR2* promoter in the presence of Hg^{2+} were approximately 4-fold higher than those in the absence of Hg^{2+}, confirmed that *PvSR2* promoter is heavy metal inducible [6]. Deletion of the entire I-PvSR from this prolonged intron-containing promoter sequence significantly reduced the GUS activity by approximately 70% and 74% in the absence and presence of Hg^{2+}, respectively (based on one-sided paired *t*-test, $P < 0.01$), indicating that I-PvSR enhanced the *PvSR2* promoter-driven *GUS* expression *in vivo*.

I-PvSR exhibits weak promoter activity

I-PvSR flanked by a short portion of exon 1 and 2 sequences drove *GUS* expression at a low level related to the *PvSR2* promoter (Fig. 2), suggesting that it functions as a weak promoter. The promoter activity of I-PvSR was further confirmed *via Agrobacterium* (carrying chimeric *I-PvSR:GUS* construct)-mediated transformation of tobacco leaf discs. The wild type tobacco control showed no GUS staining (Fig. 3A); while blue spots resulted from the I-PvSR-driven *GUS* expression were clearly visible (Fig. 3B).

The transcription start site of I-PvSR promoter is mapped within the intronic sequence

The transcription start site (TSS) of the I-PvSR-driven *GUS* transcript was mapped by 5′-RACE analysis in green calluses of transgenic tobacco. Sequence analysis of four 5′ cDNA clones revealed that each of these was 100% identical and the putative TSS located at the nucleotide (bent arrow in Fig. 1) 72 bp upstream of the 3′-splicing site.

Searching against the plant *cis-acting* elements databases, PlantCARE (http://intra.psb.ugent.be:8080/PlantCARE/), revealed several potential *cis*-acting elements in the intronic promoter region (boxed in Fig. 1). One putative auxin-response core motif [10] and three putative wound-responsive elements [11] were found. The sequence also contains one putative CAAT box at position -64 from the mapped TSS. No canonical TATA box was found.

The intronic promoter drives a shorter PvSR2 transcript by an alternative polyadenylation site usage

To investigate if I-PvSR serves as an alternative promoter, RT-PCR was performed on total RNA of bean

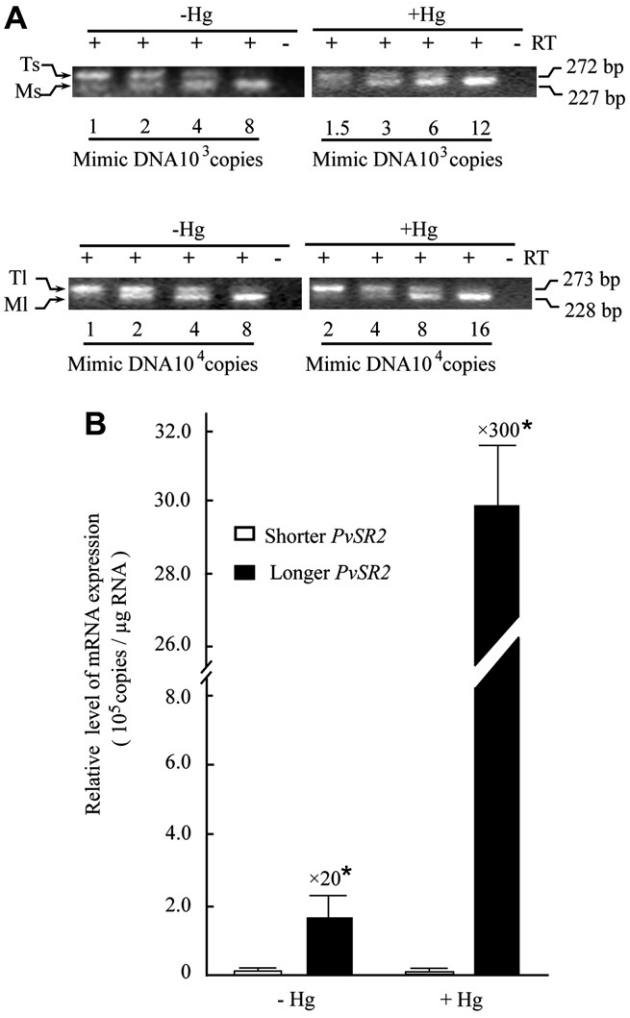

Fig. 4. Quantifying abundance of two *PvSR2* transcript species by QC-PCR. (A) Representative gel showing detection of two target *PvSR2* cDNAs (Tl for longer *PvSR2*, Ts for shorter *PvSR2*) and their mimics (Ml for longer *PvSR2*, Ms for shorter *PvSR2*). Serial dilutions of increasing known concentration of the mimic (as indicated below) were co-amplified with a constant amount of reverse-transcribed RNA(−Hg) or RNA(+Hg) using the same primers. The negative PCR was performed without prior RT reaction (−RT), indicating a lack of genomic DNA contamination. (B) The level of two *PvSR2* transcripts' expression was quantified based on QC-PCR. The level of transcript was expressed as the "number of copies/μg of total RNA". The ratio of the longer transcript abundance relative to that of shorter one is shown above the bar. Significant difference between two-transcript abundance ratios was analyzed using one-sided paired *t*-test ($^*P < 0.01$). Data represent means ± SD for three independent PCR analyses from each RT-reaction sample.

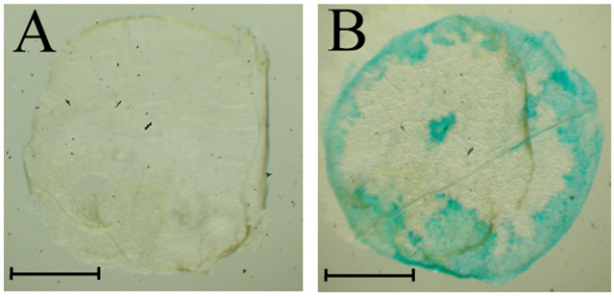

Fig. 3. Histochemical assay for I-PvSR-driven *GUS* expression in tobacco leaves. Wild type tobacco control (A) and one representative tobacco leaf disc (B) stained for GUS activity are shown. Bar = 2 mm.

leaves using primers that are complementary to the intronic sequence downstream of TSS and the poly(A) tail of this transcript. Sequencing showed that separated four positive clones were identical. This intronic promoter-driven *PvSR2* transcript is a short-form of *PvSR2* transcript (768 nt) which is 61 nt shorter than the previously defined longer form (829 nt). It begins within the intron sequence and lacks the last 56 bases of 3′-end sequence of longer native *PvSR2* transcript by utilizing an alternative polyadenylation site (vertical arrow in Fig. 1).

The longer PvSR2 transcript is more abundant than the shorter one

The relative abundance of two *PvSR2* transcripts with or without $HgCl_2$ stress was detected by QC-PCR analysis (Fig. 4A). The longer transcript was 20 and 300 times more abundant than the shorter one in the absence and presence of $HgCl_2$, respectively (Fig. 4B). The accumulation of longer *PvSR2* transcript significantly increased in response to $HgCl_2$ (15-fold of the non-induced). However, the accumulation of shorter transcript slightly decreased after $HgCl_2$ treatment. Thus, the shorter transcript is less abundant and independent of Hg^{2+}-induction, whereas the longer transcript is more predominant and strongly induced by Hg^{2+}.

Discussion

I-PvSR was able to increase the expression of the *GUS* reporter gene about 3.5-fold under control of the *PvSR2* promoter *in vivo* (Fig. 2). Thus, I-PvSR can enhance the *PvSR2* promoter-driven *PvSR2* transcription. Moreover, I-PvSR contains an alternative promoter and led to a shorter *PvSR*2 transcript with an alternative polyadenylation site (Fig. 1). Quantitatively, this shorter transcript is less abundant compared to the longer one transcribed from the upstream heavy metal-inducible promoter of the gene and is not up-regulated by Hg^{2+} treatment (Fig. 4), probably due to weak, non-metal inducible intronic promoter (Fig. 2). Thus, the *PvSR2* possesses two differentially expressed transcripts via alternative promoter and polyadenylation site usage. The production of these two *PvSR2* transcripts would require regulation of both transcriptional and posttranscriptional events, including activation of transcription and polyadenylation site selection.

Alternative promoter usage is an additional way to create regulatory diversity and provides a way for coordinating the synthesis of functionally related proteins that must act together to mediate a certain biological response [12]. In plant, there are several examples of alternative transcription from a single gene by usage of intronic promoter [13–15]. However, to our knowledge, only two examples show that an alternative promoter generates functional truncated full-size protein in plant [15,16]. Although the role of shorter transcript from *PvSR2* gene is currently unknown, there are some explanations for its function based on its putative encoding proteins. The shorter *PvSR2* transcript contains a primary ORF (142–645) and three upstream short ORFs (11–193, 31–45, and 73−193), downstream of the mapped transcription start site in the intronic sequence (Fig. 1). The primary ORF encodes a 167 amino acid truncated PvSR2 protein that lacks 33 amino acids at the N-terminus of PvSR2 protein (Fig. 1). Alternative promoter usage analyzed to date can create a full-length protein isoform but not a novel protein [12]. We also incorporated this fact to the shorter *PvSR2* transcript. Based on above analysis, a more likely possibility is that the shorter transcript may produce a truncated PvSR2 protein. In contrast, upstream short ORFs will probably have a function in regulating the translation efficiency of primary ORF [17].

A tyrosine-based sorting signal (YXXø, where ø represents a bulky hydrophobic residue) [18] is found in N-terminal 33-amino acid region (YLEF, double underlined in Fig. 1). Thus, the truncated form of PvSR2 protein that lacks sorting signal might have different subcellular localization from the full-length one. We previously showed that the truncated PvSR2 protein can enhance cadmium tolerance of *Escherichia coli* DH5α cells [5] and transgenic tobacco plants [19], suggesting that it functions as detoxification agent of heavy metal. It could therefore be hypothesized that the alternative promoter usage in the *PvSR2* gene generates two functionally related PvSR2 protein isoforms possibly involving in heavy metal detoxification. The detailed transcriptional regulation and biological function of two *PvSR2* transcripts remain to be further investigated. It should be noted that, to our knowledge, the intronic promoter identified here has not been described in a heavy metal-inducible gene. Our data can provide new insights into expression mechanism of metal inducible gene.

Acknowledgments

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