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Splicing factor SR34b mutation reduces cadmium tolerance in *Arabidopsis* by regulating iron-regulated transporter 1 gene

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ABSTRACT

Serine/arginine-rich (SR) proteins are important splicing factors. However, the biological functions of plant SR proteins remain unclear especially in abiotic stresses. Cadmium (Cd) is a non-essential element that negatively affects plant growth and development. In this study, we provided clear evidence for *SR* gene involved in Cd tolerance in planta. Systemic expression analysis of 17 *Arabidopsis SR* genes revealed that *SR34b* is the only *SR* gene upregulated by Cd, suggesting its potential roles in *Arabidopsis* Cd tolerance. Consistent with this, a *SR34b* T-DNA insertion mutant (*sr34b*) was moderately sensitive to Cd, which had higher Cd^{2+} uptake rate and accumulated Cd in greater amounts than wild-type. This was due to the altered expression of iron-regulated transporter 1 (IRT1) gene in *sr34b* mutant. Under normal growth conditions, *IRT1* mRNAs highly accumulated in *sr34b* mutant, which was a result of increased stability of *IRT1* mRNA. Under Cd stress, however, *sr34b* mutant plants had a splicing defect in *IRT1* gene, thus reducing the *IRT1* mRNA accumulation. Despite of this, *sr34b* mutant plants still constitutively expressed IRT1 proteins under Cd stress, thereby resulting in Cd stress-sensitive phenotype. We therefore propose the essential roles of SR34b in posttranscriptional regulation of *IRT1* expression and identify it as a regulator of *Arabidopsis* Cd tolerance.

1. Introduction

Serine/arginine-rich (SR) proteins are important splicing factors. These proteins are involved in many biological processes through regulating gene expression [1,2]. In mammalians, many studies have shown their essential roles in embryonic development since disruption of *SR* genes could lead to serious defects in development, such as embryonic lethal, largely due to misregulation of target gene expression [2].

Although the functions in splicing and expression patterns of plant *SR* genes have been extensively studied, their biological roles remain largely unclear [3]. Several studies have suggested that SR proteins are essential for plant development. For example, in *Arabidopsis*, overexpressing SRp30 or RSZ33 results in morphological and developmental changes [4,5]. Loss of function in SR-related protein SR45 exhibited developmental abnormalities, including delayed flowering, narrow leaves and altered number of petals and stamens [6,7]. Recently, SR45 was reported as a negative regulator of glucose and ABA signaling during early seedling development in *Arabidopsis* [8]. Of note, the biological functions of *SR* genes are not restricted to development. In some plants, such as *Arabidopsis* and rice, *SR* genes are regulated by some abiotic stresses, suggesting their potential roles in plant tolerance to abiotic stresses by regulation of downstream target genes [9,10].

Among various abiotic stresses, we focused on cadmium (Cd) because it is a non-essential element that negatively affects plant growth and development. In plants, the roots are the main site of Cd absorption and accumulation. Thus, to restrict Cd uptake into the roots is one common mechanism underlying plant Cd tolerance. In this process, Cd transporters located in plasma membrane of root epidermal cells play essential roles and the iron-regulated transporter 1 (IRT1) is believed to be one such Cd transporter [11–13]. Evidences come from the observation that overexpression of *IRT1* in transgenic *Arabidopsis* and rice (*Oryza sativa*) results in enhanced sensitivity to Cd due to accumulating high level of Cd [12,14].

In this study, using a reverse genetic approach, we demonstrate that Cd-upregulated *Arabidopsis SR34b* gene is a regulator involved in splicing, mRNA stability, and protein accumulation of *IRT1* gene,

Abbreviations: ActD, actinomycin D; Cd, cadmium; CHX, cycloheximide; IRT1, iron-regulated transporter 1; ICP-MS, inductively coupled plasma-mass spectrometry; SIET, scanning ion-selective electrode technique; SR proteins, serine/arginine-rich proteins.

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thereby participating in tolerance to Cd in *Arabidopsis* plants. These observations highlight the roles of *SR34b* in *IRT1* expression and provide clear evidence for SR protein involved in resistance to Cd in plants.

2. Materials and methods

2.1. Plant materials and growth conditions

The seeds of *Arabidopsis* (Col ecotype) were surface-sterilized and sown on 1/2 Murashige and Skoog (MS)-agar plates at 22 °C with 16-h/8-h light/dark cycles.

2.2. RNA isolation and cDNA synthesis

Total RNA was extracted from *Arabidopsis* samples using the RNAprep Pure Plant kit with on-column DNase digestion (Tiangen Biotech, Beijing, China) according to the manufacturer's protocol. RNA (2 μg) was used to synthesize the first-strand cDNA with an oligo (dT) primer according to the instruction of PrimeScript™ 1st strand cDNA synthesis kit (Takara, Dalian, China).

2.3. Identification of the sr34b knockout mutant

The *sr34b* mutant (SALK_055412), carrying a T-DNA insertion in At4g02430 of the Col ecotype, was obtained from the Arabidopsis Biological Resource Center. RT-PCR was used to verify homozygous T-DNA insertion for *SR34b* using E2 (5′-GATTTACGTCGGGAAC CTTC-3′) and E12 (5′-TTACTTGCTTCCTTCTGAAC-3′).

2.4. Cd tolerance assay

The seeds of wild-type and *sr34b* were sowed on a 1/2 MS agar medium without (control) and with 50 μM $CdCl_2$. The root length was measured at 2 weeks after sowing. To measure Cd content in plants, four-week-old wild-type and *sr34b* seedlings were transferred onto filter paper soaked with 1/2 MS liquid medium containing 25 μM $CdCl_2$ for 2 weeks. The culture medium was changed every 3 days. Shoots and roots were harvested separately, washed three times with ice-cold water, and then digested with 11 N HNO_3 at 200 °C overnight. Digested samples were diluted with 0.1 N HNO_3 and Cd content was analyzed using an inductively coupled plasma-mass spectrometry (ICP-MS) at Beijing center for physics and chemical analysis.

2.5. Expression pattern analysis of SR genes under Cd stress

Two-week-old wild-type *Arabidopsis* seedlings were transferred onto a filter paper soaked with 1/2 MS liquid without (control) or with 50 μM $CdCl_2$ for 8 h. The semi-quantitative RT-PCR analysis of 17 *SR* genes was performed using the primers previously described [9]. *TUA4* (At1g04820) was used as an internal RT-PCR control using 5′-CTCTACCTCCGTTGTTGAGCCTTAC-3′ and 5′-CACCCACATA CCAGTGAACGAAAG-3′.

2.6. Quantitative RT-PCR analysis

Two-week-old wild-type *Arabidopsis* seedlings were transferred onto a filter paper soaked with 1/2 MS liquid without or with 100 μM $CdCl_2$ for 24 h. Quantitative RT-PCR analysis of selected genes was performed on a Rotor-gene 6000 instrument (Rcorbett Research, Australia) using SYBR premix Ex-Taq kit (Takara, Dalian, China) according to manufacturer's instructions. *YLS8* (At5g08290) was used as an internal control and relative expression levels were analyzed using a $2^{-\Delta\Delta Ct}$ method. The thermal cycling conditions were 95 °C for 10 min, 50 cycles of 95 °C for 30 s, 55 °C for 30 s and 72 °C for 20 s. The primers used were as follows: 5′-GGTTGTATCCTCCAGGCTGAGT-3′ and 5′-GCATTTAGAAGTCCAACCG TGAT-3′ for *IRT1* (At4g19690), 5′-TTACTGTTTCGGTTGTTCTCCATTT-3′ and 5′-CACTGAATCATGTTCGAAGCAAGT-3′ for *YLS8*.

2.7. Intron splicing RT-PCR analysis

Two-week-old wild-type *Arabidopsis* seedlings were transferred onto a filter paper soaked with 1/2 MS liquid without (control) or with 100 μM $CdCl_2$ for 24 h. RT-PCR splicing analysis of selected genes was performed using following primers: 5′-TCAGCACTTCT-CATGAAAACAATC-3′ and 5′-GAGATGCAAAAACATTACACGATAT-3′ for *IRT1*, 5′-CTTTTGCTTCTCGTTACGTCCG-3′ and 5′-CTCGTTGTGG CGGCTACTATCT-3′ for *GAD* (At2g02010), 5′-GGTTTATGAGGGAA TCTGAGTTCC-3′ and 5′-CTCCAGGGCACATAAATCCATC-3′ for *APS3* (At4g14680), 5′-TGGAAAGGGTTATCCTAATTCCC-3′ and 5′-AACA GCGATTTGAACAGTCTTAGG-3′ for *HSP17.4-CIII* (At1g54050), 5′-CGTCTCGTCGTCATTCGTTTC-3′ and 5′-CCAGTTCACGGGATATTT-CAGC-3′ for *YLS8*. Genomic DNA was used as a positive PCR control. PCR was performed on 1 μL of the resulting cDNA using the 2 × *Taq* PCR StarMix with loading dye (GenStar Biosolutions Co. Ltd.). The amplification began with 94 °C for 4 min, followed by 25–35 cycles of 94 °C for 30 s, 55–60 °C for 30 s and 72 °C for 60 s with a final elongation at 72 °C for 7 min. The RT-PCR products were cloned into the pMD18T-vector (TaKaRa, Dalian, China) and sequenced.

2.8. Western blot of IRT1

Four-week-old wild-type and *sr34b* seedlings were transferred onto filter paper soaked with 1/2 MS liquid medium without or with 100 μM $CdCl_2$ for 2 days. Total protein was extracted from 1 g of root tissues grown in the conditions described above, as previously described [12,13]. Proteins separated on a SDS–PAGE (12%) were electrophoretically transferred to a pure nitrocellulose blotting membrane (Pall Life Sciences, Pensacola, Fla.) and probed with the primary anti-IRT1 rabbit peptide (PANDVTLPIKEDDSSN) antiserum (1:400 diluted, Beijing Protein Innovation, China) as previously described [12,13]. The secondary antibody used was HRP-linked anti-rabbit IgG (Cell Signaling Technology) (diluted 1:5000 in blocking buffer). Chemiluminescence was performed on a Fujifilm LAS-4000mini imager with ECL Prime Western Blotting detection reagent (Amersham Biosciences). The non-specific signal is used as a loading control as described [15].

2.9. Cd^{2+} flux measurements by scanning ion-selective electrode technique (SIET)

After exposure to 50 μM $CdCl_2$ for 10 min, the roots of 4-day-old wild type and *sr34b* seedlings were sampled for the Cd^{2+} flux measurement. The net Cd^{2+} flux was measured noninvasively using SIET (BIO-001A; Younger USA, Amherst, MA, ScienceWares, Falmouth, MA.) as previously described [16].

2.10. mRNA stability assay

Six-week-old wild-type and *sr34b* seedlings were transferred to plates with 1/2MS medium containing 0.6% agar, 1 mM MES, and 300 μM Ferrozine ([3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine sulfonate], a strong iron chelator) for 3 days. The roots were harvested and cut into sections (about 5 mm) and used for inhibitor treatment. Root sections were then suspended in a 10-fold dilution of MS medium containing 20 μM cycloheximide (CHX, Sigma) and 100 μg/mL Actinomycin D (ActD, Sigma), as previously described [17]. The root sections were vacuum-infiltrated for 10 min with the inhibitors and incubated at room temperature. The samples

were removed from the cultures and frozen in liquid nitrogen at 2-h intervals. RNAs were then extracted and subjected to qRT-PCR using *YLS8* as an internal control. *IRT1* mRNA abundance was expressed as a ratio relative to pretreatment levels (0 h), which was set to a value of 1.0.

3. Results

3.1. Eight Arabidopsis SR genes respond to Cd stress

We employed RT-PCR analysis to measure the expression of *SR* genes in *Arabidopsis* upon Cd exposure. Results showed that the mRNA level of seven *SR* genes (*RS31*, *RS40*, *SRZ22*, *RSZ32*, *SCL30*, *SCL30a*, and *SCL28*) was reduced; whereas the *SR34b* mRNA level was induced (Fig. 1). We selected *SR34b* for further study because it is the only gene upregulated by Cd among of 17 *SR* genes.

3.2. SR34b T-DNA insertion mutant is sensitive to Cd

To explore the physiological relevance of *SR34b* in *Arabidopsis* response to Cd, we established a line homozygous T-DNA insertion into *SR34b* locus (Fig. 2A and B) and measured Cd tolerance using this *sr34b* mutant. Under normal growth condition, the root length of *sr34b* mutant was significantly longer than that of wild-type control. In the presence of Cd, however, *sr34b* mutant showed a root growth retardation phenotype (Fig. 2C). This indicated that *SR34b* mutation sensitizes root growth to Cd stress. Furthermore, Cd content analysis revealed that *SR34b* mutation led to moderate accumulation of Cd compared with wild-type. The shoots and roots of *sr34b* mutants accumulated Cd approximate 78% and 50% higher than those of wild-type plants (Fig. 2D), respectively. These results suggested that *SR34b* mutation results in a moderate sensitivity to Cd.

3.3. sr34b has a higher Cd^{2+} uptake rate than wild-type

High Cd^{2+} uptake activity of *sr34b* mutant may contribute to its high level of Cd accumulation. To test this hypothesis, we measured Cd^{2+} influx of root hairs of *sr34b* mutant and wild-type seedlings by SIET. Although both wild-type and *sr34b* were able of taking up Cd^{2+} (Fig. 3A), *sr34b* root hairs exhibited a 4.38-fold higher Cd^{2+}influx than those of wild-type (Fig. 3B). This result indicated that *sr34b* has a higher Cd^{2+} uptake rate than wild-type.

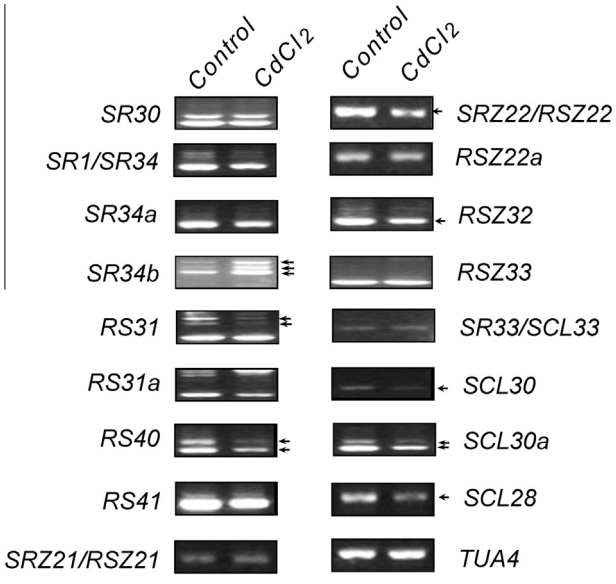

Fig. 1. Characterization expression of *Arabidopsis SR* genes upon Cd exposure. Two-week-old *Arabidopsis* seedlings were exposed to 50 μM $CdCl_2$ for 8 h and then subjected to RT-PCR analysis. *TUA4*, encoding a α-tubulin isoform, served as a loading control. Arrows indicate the transcripts that are regulated by Cd stress. Experiments were repeated at least twice showing similar results.

3.4. sr34b mutant plants have a splicing defect in IRT1 gene under Cd stress

IRT1 is responsible for Cd^{2+} uptake in roots [11]. The high level of Cd^{2+} uptake capacity of *sr34b* may be due to the altered *IRT1* expression. We therefore examined whether the *IRT1* expression in *sr34b* by quantitative RT-PCR analysis. The *IRT1* mRNA level was significantly upregulated by 5-fold in *sr34b* mutant (Fig. 4A). However, we did not detect significant change of *IRT1* expression when *sr34b* mutant and wild-type were treated with Cd, suggesting that Cd stress eliminates this upregulation of *IRT1* in *sr34b* mutant (Fig. 4A). This was due to the defect in *IRT1* splicing in *sr34b* under Cd stress (Fig. 4B).

3.5. SR34b mutation stabilizes IRT1 mRNAs

Chicken ASF/SF2, an ortholog of SR34b in animals, controls its downstream target *PKCI-1-related* mRNA stability [18]. We further examined whether the *IRT1* mRNA stability is affected in *sr34b* mutant plants. No or little *IRT1* mRNA is detectable in *Arabidopsis* under normal growth conditions [12]. This can result in technically difficult to detect the decay rate of *IRT1* mRNAs. We therefore used the iron-deficient roots of wild-type and *sr34b* mutant to perform *IRT1 mRNA* stability assay. The results demonstrated that the decay rate of *IRT1* mRNAs was significantly faster in wild-type than *sr34b* (Fig. 4C), indicating that the *IRT1* mRNAs were stabilized by *SR34b* mutation. Thus, enhanced *IRT1* mRNA stability may partially contribute to the increasing *IRT1* mRNA accumulation observed in *sr34b*.

3.6. SR34b mutation leads to constitutive accumulation of IRT1 proteins

Arabidopsis IRT1 protein is expressed in the roots when iron is limiting [12]. This raises a possibility that IRT1 protein cannot accumulate under Cd stress. By using the immunoblotting assay, we found that IRT1 protein was not detected in Cd-stressed wild-type plants but was presented in *sr34b* mutant even under Cd stress (Fig. 4D). This suggests that *SR34b* mutation leads to constitutive accumulation of IRT1. Hence, constitutive accumulation of IRT1 protein in Cd-stressed *sr34b* mutant may partially contribute to its high level of Cd^{2+} uptake capacity, although *IRT1* mRNA level was not significantly altered by Cd in *sr34b* (Fig. 4A).

4. Discussion

In this study, we used a reverse genetic approach to demonstrate that mutation in *SR34b* causes a moderate decrease in Cd tolerance. *sr34b* mutant plants have higher Cd^{2+} uptake rate and accumulated Cd in greater amounts than wild-type. The constitutive accumulation of IRT1 protein in *sr34b* is responsible for Cd stress-sensitive phenotype of this mutant. Although disruption of *SR34b* inhibited the *IRT1* splicing and then reduced its mRNA level under Cd stress, IRT1 protein accumulation still occurred in *sr34b* mutant and enabled it to take up and accumulate more Cd than wild-type. This is consistent with a previous study showing that constitutive accumulation of IRT1 protein in transgenic *Arabidopsis* grown in iron-deficient medium results in enhanced sensitivity to Cd due to accumulating more Cd [12].

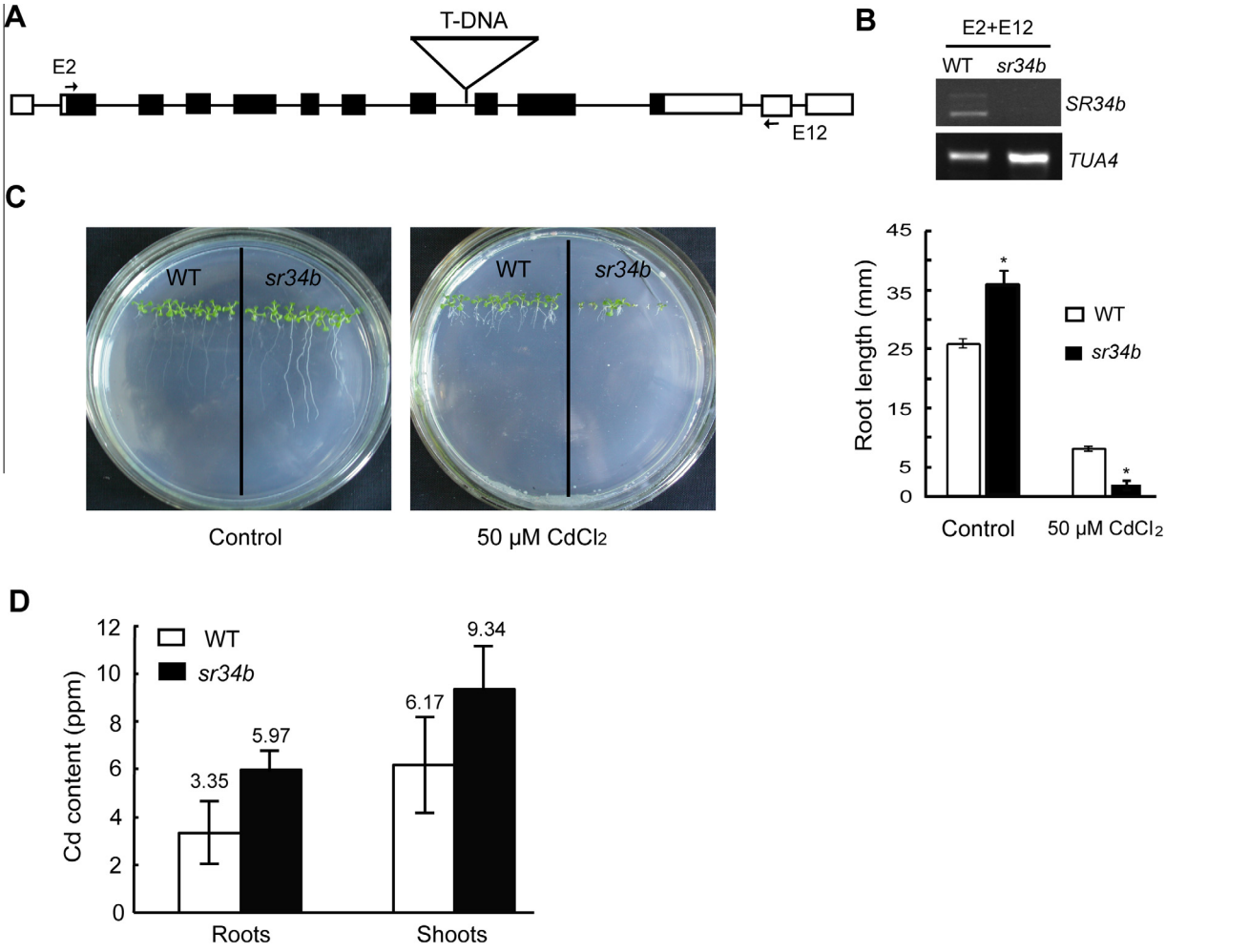

Fig. 2. *sr34b* mutant is sensitive to Cd stress. (A) The T-DNA was inserted in the eighth intron of the *SR34b* genomic DNA. Open boxes and lines denote exons and introns, respectively. Filled boxes represent coding region. (B) RT-PCR identification of wild-type and *sr34b* plants using the exon 2-specific forward primer (E2, shown in Fig. 2A) and the exon 12-specific reverse primer (E12, shown in Fig. 2A). *TUA4* served as a loading control. (C) The seeds of *sr34b* and wild-type were sowed on a 1/2 MS agar medium without (control) and with $CdCl_2$. The root length was measured at 2 weeks after sowing. The data represent means ± SD ($n = 8$). Asterisks indicate significant differences ($^*P < 0.01$) between *sr34b* and WT control. (D) Four-week-old *sr34b* and wild-type plants were grown on 1/2 MS culture medium containing 25 μM $CdCl_2$ for 2 weeks. The roots and shoots were harvested separately and subjected to ICP-MS. Approximately 100 plants were pooled for each experiment, and results are means of three independent experiments. Bars indicate SD.

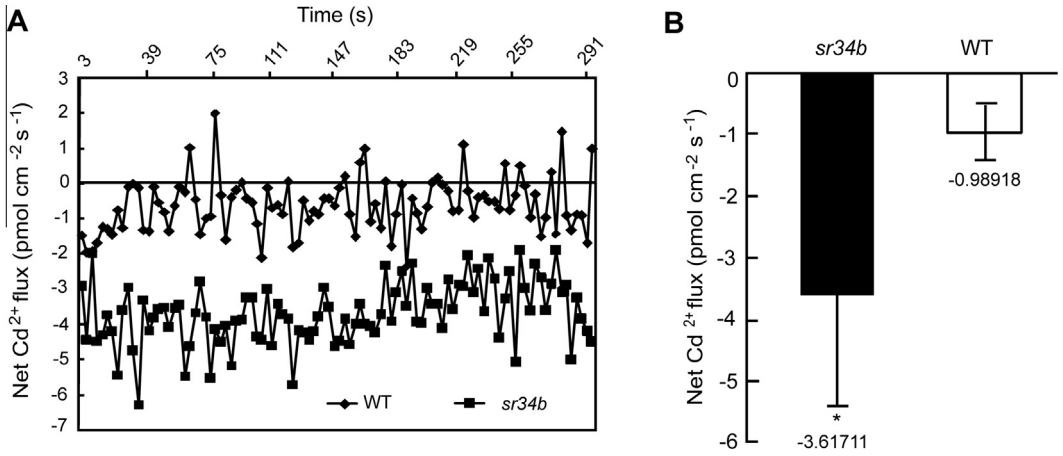

Fig. 3. SIET assay showing that *sr34b* has a higher Cd^{2+} uptake rate than wild-type. (A) Net Cd^{2+} fluxes in the roots of wild-type (WT) and *sr34b* seedlings within measuring periods (300 s). Each point represents the mean of five to seven individual roots. (B) The mean fluxes of Cd^{2+} within the measuring periods. The bars represent SD. Asterisks indicate significant differences ($^*P < 0.05$) between *sr34b* and WT control.

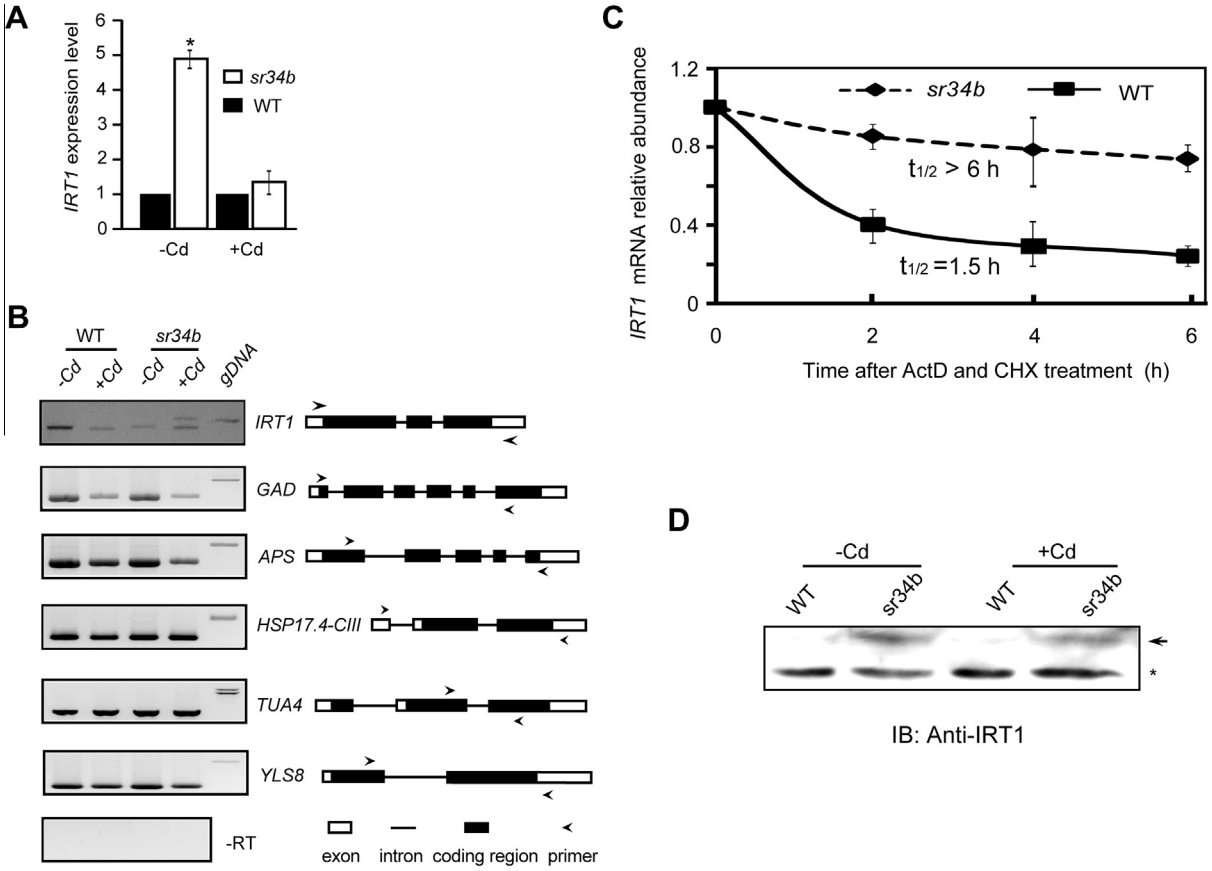

Fig. 4. Charateriation of *IRT1* expression *in sr34b* mutant. Two-week-old wild-type and *sr34b* seedlings were treated without (−Cd) or with 100 μM $CdCl_2$ (+Cd) for 24 h and then subjected to qRT-PCR (A) and RT-PCR splicing analysis (B) of *IRT1*. Data are means ± SD of three independent experiments (*t-test significant at $P < 0.05$). gDNA, genomic DNA control. "−RT", reactions without reverse transcriptase. The lack of bands in the −RT reaction demonstrated no genomic DNA contamination. For *IRT1*, the upper band corresponds to the unspliced form (as in gDNA) and the lower band to the fully spliced form. The structure of the gene is shown on the right panel. (C) *SR34b* mutation stabilizes the *IRT1* mRNAs. The iron-starved roots of wild-type and *sr34b* plants were treated with a transcription inhibitor ActD and a protein synthesis inhibitor CHX for 0, 2, 4, and 6 h. RNAs were then extracted and subjected to qRT-PCR. The constitutively expressed *YLS8* mRNA, which has a half-life more than 19 h [30], was used as an internal control. *IRT1* mRNA abundance was expressed as a ratio relative to pretreatment levels (0 h), which was set to a value of 1.0. Data shown are the average of three independent qRT-PCR experiments for each time point. Error bars, SD. The half-life time ($t_{1/2}$) of *IRT1* mRNA in WT and sr34b samples was shown. (D) Immunoblot analysis showing constitutive expression of IRT1 protein in *sr34b*. Total proteins were extracted from roots of 4-week-old wild-type and *sr34b* plants grown in 1/2 MS liquid medium(−Cd) or same medium containing 100 $CdCl_2$ (+Cd) for 2 days and subjected to immunoblot (IB) with anti-IRT1antiserum. The asterisk labels a non-specific signal that is used as a loading control as described [15]. The IRT1 protein signal is indicated by an arrow. Experiments were repeated at least twice showing similar results.

In fact, *sr34b* mutant only shows a moderate Cd-sensitive phenotype, suggesting a possible functional redundancy of SR34b and other SR proteins in Cd tolerance of *Arabidopsis*. In addition to *SR34b*, other seven *SR* genes also respond to Cd stress (Fig. 1), indicating they may be involved in Cd tolerance of *Arabidopsis* plants. Hence, it would be of interest to generate double/triple mutants of these eight Cd-responsive SR genes and thus analyze their Cd tolerance functions.

Previous studies have shown that *IRT1* is subject to posttranscriptional control [12,13]. Several lines of evidence established in this study imply an important role for SR34b in posttranscriptional regulation of *IRT1* expression. First of all, *sr34b* mutant plants constitutively express IRT1 protein under normal growth and Cd stress conditions. Secondly, *sr34b* mutant plants have a splicing defect in *IRT1* upon Cd stress. Thirdly, SR34b mutation stabilizes *IRT1* mRNAs.

How SR34b regulates constitutive accumulation of IRT1 protein is unknown. More recently, several studies have suggested that IRT1 protein degradation is regulated by ubiquitination at least partially through a RING domain-containing E3 Ubiquitin Ligase IDF1 [15,20,21]. Several transcription activators of *IRT1*, FIT, bHLH38, bHLH39, bHLH100, and bHLH101, have been proved to be involved in regulating IRT1 protein accumulation [22–24]. It will be great interest to investigate the potential roles of SR34b in these regulatory pathways of IRT1 protein accumulation.

More interestingly, SR34b is essential for *IRT1* splicing under Cd stress, because intron retention of *IRT1* occurred in Cd-stressed *sr34b* mutant but not in Cd-stressed wild-type plants (Fig. 4B). To test whether this intron retention is due to a widespread toxicity of Cd on RNA splicing, we examined three Cd-inducible, intron-containing genes (*GAD*, *APS3*, and *HSP17.4-CIII*) [19] and two constitutively expressed genes (*TUA4* and *YLS8*) and found that splicing of these genes were unaffected in Cd-treated *sr34b* plants as well as other plant samples (Fig. 4B). Therefore, this inhibition of splicing by Cd appears to be specific for *IRT1* at least for five genes tested here. Similar to *IRT1*, inhibition of splicing by Cd have been observed in three plant Cd-responsive genes, including petunia *hsp70* gene [25], maize *Bz2* gene [26], and soybean *Gmhsp26-A* gene [27,28].

Although the mechanism by which Cd interferes with the splicing process of gene is largely unknown, some specific splicing-related factors may involve in Cd-dependent intron retention. Our study about *IRT1* splicing in *sr34b* background suggested that SR34b may be a candidate splicing factor required for Cd-dependent intron retention. Several lines of evidence established in this work support our conclusion. First of all, *SR34b* expression is upregulated

by Cd stress. Secondly, *sr34b* is Cd sensitive. Thirdly, *sr34b* is defective in the splicing of *IRT1* under Cd stress. How SR34b transfers Cd signal to regulate the *IRT1* splicing needs to be elucidated. Similar to SR34b, *Arabidopsis* STABILIZED1 (STA1) is one splicing factor responsible for stress-dependent intron retention [29]. *sta1-1* mutant is sensitive to cold and osmotic stresses and defective in splicing of the cold-induced *COR15A* gene. This intron inhibition occurs in under cold stress but not under ABA or NaCl stress.

SR34b may be a negative factor involved in *IRT1* mRNA stability, because the decay rate of *IRT1* mRNAs was significantly faster in wild-type than in *sr34b* (Fig. 4C). Further studies determine whether SR34b directly binds to the *IRT1* mRNA will be of interest, which allows us to distinguish direct or indirect regulation of *IRT1* mRNA stability by SR34b.

In conclusion, we provide clear evidence that splicing factor SR34b is required for Cd tolerance through regulating the splicing and stability of downstream target *IRT1* gene and enhancing constitutive accumulation of IRT1 proteins. These observations highlight the roles of SR proteins in Cd tolerance of plants.

Acknowledgments

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