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Arabidopsis exoribonuclease USB1 interacts with the PPR-domain protein SOAR1 to negatively regulate abscisic acid signaling

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Abstract

Signaling by the phytohormone abscisic acid (ABA) involves pre-mRNA splicing, a key process of post-transcriptional regulation of gene expression. However, the regulatory mechanism of alternative pre-mRNA splicing in ABA signaling remains largely unknown. We previously identified a pentatricopeptide repeat protein SOAR1 (suppressor of the ABAR-overexpressor 1) as a crucial player downstream of ABAR (putative ABA receptor) in ABA signaling. In this study, we identified a SOAR1 interaction partner USB1, which is an exoribonuclease catalyzing U6 production for spliceosome assembly. We reveal that together USB1 and SOAR1 negatively regulate ABA signaling in early seedling development. USB1 and SOAR1 are both required for the splicing of transcripts of numerous genes, including those involved in ABA signaling pathways, suggesting that USB1 and SOAR1 collaborate to regulate ABA signaling by affecting spliceosome assembly. These findings provide important new insights into the mechanistic control of alternative pre-mRNA splicing in the regulation of ABA-mediated plant responses to environmental cues.

Keywords: Abscisic acid signaling, Arabidopsis thaliana, early seedling growth, exoribonuclease USB1, pentatricopeptide repeat (PPR) protein SOAR1, spliceosome assembly.

Introduction

Post-transcriptional regulation plays crucial roles in the life cycle of eukaryotes (Halbeisen et al., 2008). As a key process of this regulation, pre-mRNA splicing involves excising introns and joining together exons to form the mature mRNA. This process is catalyzed by the spliceosome, a large molecular complex consisting of five small nuclear ribonucleoprotein particles (snRNPs) and a multitude of non-snRNP proteins in human and yeast. The snRNPs are composed of small nuclear uridine-rich RNAs (U1, U2, U4, U5, and U6 snRNAs) and their corresponding interacting proteins (Jurica and Moore, 2003; Will and Luhrmann, 2011; Fica and Nagai, 2017). The splicing reaction is initiated by the recruitment of U1snRNP to the 5' splice site (5' SS) of pre-mRNA. Splicing factor 1 and the 35 kDa U2 auxiliary factor subunit bind to the branch point (BP) sequence and the polypyrimidine tract of the intron, respectively, which aids U2 snRNP binding to form the pre-spliceosome (A complex), and further recruits the U4/U6-U5 tri-snRNP to generate the pre-catalytic complex B. The B complex is assembled into the B* complex, which further reorganizes into the B** complex. The B* complex catalyzes...
the first of the transesterification reactions. This process generates complex C, which causes a cleaved 5’ SS and lariat intron. Further, the conversion of the complex to C* promotes the second catalytic step, resulting in cleavage of the 3’ splice site (3’ SS) and formation of a post-spliceosomal complex. Finally, exons are joined together to form the mature mRNA, introns are released, and snRNPs are recycled (Will and Luhrmann, 2011; Fica and Nagai, 2017; Shi, 2017). It is particularly noteworthy that the intramolecular stem–loop of U6 snRNA, the helix I of the U2/U6 duplex, loop I of U5 snRNA, and the catalytic magnesium ions are components of the core active site of the spliceosome (Shi, 2017).

In plants, the process by which the spliceosome is assembled is still unclear. Recent studies, however, revealed various regulatory mechanisms of alternative splicing. Chromatin structure, RNA polymerase II elongation rate, serine/arginine-rich proteins, and heterogeneous nuclear RNPs (hn RNP) may be involved in the recognition of splicing sites and spliceosome activity (Luco et al., 2011; Koncz et al., 2012; Brzyszek and Swieczewski, 2015; Nieto Moreno et al., 2015; Romero-Barrios et al., 2018). Differences may exist in splicing machineries and sequence recognition between plants and animals. The average size of introns in animals (~5 kb) is longer than that in plants (~160 bp), but the consensus sequences of the 5’ SS, 3’ SS, and BP are similar in animals and plants (Simpson et al., 2002; Sakharkar et al., 2004; Iwata and Gotoh, 2011). Alternative splicing, which generally includes five categories of events, namely intron retention, exon skipping, 5’ SS alternative splicing, 3’ SS alternative splicing, and mutually exclusive exon splicing, generates multiple mature mRNA isoforms from the same pre-mRNA. There are ~95% of intron-containing genes in humans and ~61% of intron-containing genes in Arabidopsis thaliana that are alternatively spliced, whereas the most frequent alternative splicing event in human is exon skipping but in plants is intron retention (~40%) (Pan et al., 2008; Marquez et al., 2012; Reddy et al., 2013).

The phytohormone abscisic acid (ABA) regulates multiple aspects of plant development and plant adaption to environmental stress (reviewed in Finkelstein et al., 2002; Adie et al., 2007; and Cutler et al., 2010). There is mounting evidence that alternative splicing plays a crucial role in ABA signaling (reviewed in Laloum et al., 2018). HAB1 is a member of the clade-A type-2C protein phosphatases (PP2Cs), which negatively regulates ABA signaling (Umezawa et al., 2009; Vlad et al., 2009). The HAB1 pre-mRNA undergoes alternative splicing to produce two functional splice variants HAB1.1 and HAB1.2 that function oppositely to regulate ABA signaling (Z. Wang et al., 2015; Zhan et al., 2015). The ROA1/RBM25 (Regulator of ABA Response), a homolog of human splicing factor RBM25, regulates alternative splicing of HAB1 and numerous other genes that participate in ABA signaling (Z. Wang et al., 2015; Zhan et al., 2015). The two mRNA cap-binding proteins CBF20 and CBF80 function as negative regulators of ABA responses by influencing alternative splicing of the first intron in most genes, particularly at the 5’ SS in Arabidopsis (Hugouvieux et al., 2001; Papp et al., 2004; Raczyńska et al., 2010). Additionally, two ABA-responsive genes, ABI5 and CIPK3, also undergo alternative pre-RNA splicing by which these two genes function to affect ABA signaling (Zou et al., 2007; Sanyal et al., 2017a; Xiong et al., 2019).

USB1 (U6 biogenesis protein 1) is a 3’-5’ exoribonuclease belonging to the 2H phosphodiesterase superfamily that shortens the poly(U) tail of U6 snRNA in most eukaryotes including human, yeast, and maize (Mroczek et al., 2012; Schepachev et al., 2012; Hilkenko et al., 2013; Didychuk et al., 2017; Li et al., 2017). In humans, processing of U6 by USB1 creates a terminal 2’, 3’-cyclic phosphate which stimulates binding of U6 to a subunit of the spliceosome, the Sm-like (LSm) proteins 2–8, to form the U4/U6 snRNPs (Licht et al., 2008; Hilkenko et al., 2013; Didychuk et al., 2016). The Arabidopsis homologues of human LSm, namely LSm2–LSm8, form a complex where LSm8 is a component of the U6 snRNP and were shown to be required for precursor mRNA splicing through U6 snRNA stabilization, which plays an important role in the environment-dependent regulation of spliceosome activity (Perea-Resa et al., 2012; Carrasco-Lopez et al., 2017; Huertas et al., 2019). The LSm1–LSm7 complex was reported to differentially regulate Arabidopsis tolerance to abiotic stress conditions by promoting selective mRNA decapping and especially by targeting to NCED3 and NCED5, two key enzymes in ABA biosynthesis (Perea-Resa et al., 2016). The Arabidopsis LSm4 and LSm5 were reported to function in ABA and salt stress responses by affecting alternative splicing (Xiong et al., 2001; Zhang et al., 2011; Cui et al., 2014). In maize, a loss-of-function mutation in the USB1 gene results in abnormal 3’ ends of U6 snRNAs and splicing defects of many genes, affecting seed development (Li et al., 2017). However, the regulatory mechanism of alternative pre-mRNA splicing by which plants regulate ABA signaling remains largely unknown.

We previously identified the pentatricopeptide repeat (PPR) protein SOAR1 (for Suppresser of the ABAR–overexpressor 1) as a crucial player (Mei et al., 2014; Jiang et al., 2014, 2015) downstream of the ABAR (the putative ABA receptor; Shen et al., 2006; Wu et al., 2009) in ABA signaling. In this study, we identified a SOAR1 interaction partner, USB1, which is an exoribonuclease catalyzing U6 production, and revealed that USB1 cooperates with SOAR1 to negatively regulate ABA signaling in early seedling development, probably by affecting spliceosome assembly. USB1 and SOAR1 are both required for the splicing of transcripts of many genes, including those involved in ABA signaling and salt response pathways. These findings reveal the mechanistic basis for alternative pre-mRNA splicing in the regulation of ABA-mediated plant responses to environmental cues.

Materials and methods

Plant materials and growth conditions

The Arabidopsis Columbia-0 (Col-0) ecotype was used as the wild type and all mutant background materials. The T-DNA insertion line of the usb1-1 (SAIL_717_G03) mutant in the USB1 gene (Arabidopsis genomic locus At5g51710) was obtained from the Arabidopsis Biological Resource Center (ABRC). The usb1-2 mutant was generated using CRISPR/Cas9 [clustered regularly interspaced palindromic repeats (CRISPR/Cas)].
USB1 regulates ABA signaling

Floral infiltration into Arabidopsis wild-type (Col-0) plants. T1 transgenic plants were screened by hygromycin resistance and confirmed by DNA sequencing. The homozygous T2 generation seeds were selected for further analysis. The seeds of the soar1-2 mutants were obtained from the Versailles Genetics and Plant Breeding Laboratory, Arabidopsis thaliana Resource Centre (INRA) and identified as a knockdown allele as described previously (Mei et al., 2014; Jiang et al., 2015). The primers for identification of these mutants are listed in Supplementary Table S1 at JXB online.

To generate transgenic lines overexpressing the USB1 gene, the ORF sequence of the USB1 gene was amplified by PCR and cloned into the binary vector PMDC85 with a green fluorescent protein (GFP) tag under control of the Cauliflower mosaic virus (CaMV) 35S promoter. The resulting plasmid (USB1-PMDC85) was introduced into A. tumefaciens strain GV3101 and transformed by floral infiltration into Arabidopsis wild-type (Col-0) plants. Transgenic plants with a single T-DNA insertion were screened by hygromycin resistance and confirmed by quantitative real-time PCR (qRT-PCR). T1 generation seeds of two homozygous overexpression lines, OE1 and OE3, were selected for further analysis. The above plasmid (USB1-PMDC85) was also transformed into usb1-1 and usb1-2 mutants to generate complementation lines. All of the primers used for generation of the transgenic plants are presented in Supplementary Table S1.

The usb1-1 soar1-2 and usb1-2 soar1-2 double mutants were generated by genetic crossing and identified by PCR genotyping. For the generation of the USB1overexpression plant in the soar1-2 mutant background, the overexpressed USB1 gene was introduced into soar1-2 by crossing the USB1-OE3 line and soar1-2 plants. For the generation of the SOAR1 overexpression plant in the usb1 mutant background, the previously generated SOAR1 overexpression line OE6 (expressing SOAR1 fused with GFP; Mei et al., 2014; Jiang et al., 2015) was used, and this overexpressed SOAR1 in SOAR1-OE6 was introduced to usb1-1 and usb1-2 by crossing. Plants were grown in a growth chamber at 22 °C on Murashige and Skoog (MS) medium (MS salt, 2% sucrose, 0.8% agar, pH 5.7–6.0) in a growth chamber at ~80 μmol photons m⁻² s⁻¹ or in compost soil at ~120 μmol photons m⁻² s⁻¹ lit by cool-white fluorescent lamps under a 16 h light/8 h dark photoperiod and 60% relative humidity.

Bimolecular fluorescent complementation assay in Arabidopsis protoplasts and tobacco leaves

The bimolecular fluorescent complementation (BiFC) assays were performed essentially as previously described (Shang et al., 2010). Yellow fluorescent protein (YFP) was used for the BiFC assays. The coding region of the USB1 or SOAR1 gene was cloned into the pUC-SPYNE vector (Walter et al., 2004) harboring the N-terminal half of YFP (NYFP) or the C-terminal half of YFP (CYFP) to form USB1–NYFP and USB1–CYFP, or SOAR1–NYFP and SOAR1–CYFP. The resulting plasmids were co-transformed into Arabidopsis wild-type (Col-0) mesophyll protoplasts by the polyethylene glycol-mediated transient transformation protocol (Yoo et al., 2007). The YFP fluorescence was imaged under a confocal laser scanning microscope (Zeiss LSM780, Germany).

To study the subcellular localization of the interaction between SOAR1 and USB1, we performed the BiFC assay in tobacco (N. benthamiana) leaves as described previously (Jia et al., 2016). The coding regions of USB1 and SOAR1 were fused to the NYFP and CYFP, respectively, to form USB1–NYFP and SOAR1–CYFP. The resulting plasmids were transformed into A. tumefaciens strain GV3101, and infiltrated into young but fully expanded leaves of 7-week-old tobacco leaves using a needleless syringe. After infiltration, plants were grown in the dark for 24 h and then under a 16 h light/8 h dark photoperiod for 48–60 h. The YFP fluorescence was imaged under a confocal laser scanning microscope (Zeiss LSM780, Germany). The primers used for constructing the related plasmids are listed in Supplementary Table S1.

Co-IP assay

The Co-IP assay was performed as previously described (Shang et al., 2010). Total proteins of transgenic plants (USB1–OE3) expressing USB1–GFP fusion protein were extracted, with the extraction buffer consisting of 50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% (v/v) TritonX-100, 10% (v/v) glycerol, 1X protease inhibitor cocktail (Roche, 4 693 159 001, Germany), 1 mM phenylmethylsulfonyl fluoride (PMSF), and 1 mM DTT. The total proteins were incubated with the anti-GFP mAb-Magnetic Beads (MBL, D153-11, Japan) with rotating at 4°C for 12 h. The beads were collected and washed six times with the pre-cooled extraction buffer and then re-suspended with 2 vol of the protein loading buffer containing 100 mM Tris–HCl (pH 6.8), 4% (v/v) SDS, 0.1% (w/v) bromophenol blue, 20% (v/v) glycerol, and 5% (v/v) β-mercaptoethanol. The immunoprecipitates and the input were separated on a 10% SDS-PAGE gel, immunoblotted with the anti-SOAR1 or anti-GFP antibodies, and detected using Clarity™ Western ECL Substrate (Bio-Rad, 170-5104, USA). The primers used for the vector constructions are shown in Supplementary Table S1.

In vitro pull-down assay

The full-length coding regions of SOAR1 (linked to His) and USB1 (linked to GST) were amplified by PCR, and cloned into the pMAL-c5X vector (NEB, N8108S, USA) and pGEX4-T-1 (Pharmacia) vector, respectively. The constructions were transformed into Escherichia coli BL21 (DB3) to produce SOAR1-His, USB1–glutathione S-transferase (GST), or GST protein. The GST and USB1–GST proteins were purified from E. coli using glutathione beads 4FF (Smart-life sciences, SA10H001, China) according to the manufacturer’s instructions, and the SOAR1–His proteins were purified using Ni Sepharose 6 Fast Flow agarose (GE,
17:5318-06, USA). The USB1–GST and GST were incubated with glutathione–agarose beads (Smart-life sciences, M00201, China) rotating at 4°C for 2 h in incubation buffer consisting of 50 mM Tris–HCl (pH 7.5), 50 mM NaCl, 1% (v/v) glycerol, 0.1% (v/v) Tween-20, 0.15% (v/v) β-mercaptoethanol, 25 mM imidazole, and 1% protease inhibitor cocktail. The beads were collected and washed three times with incubation buffer on ice. Then, the SOAR1-His fusion protein was incubated with the above–washed beads in the incubation buffer rotating at 4°C for another 2 h. The beads were collected and washed six times with the incubation buffer and re-suspended in 2 vols of the protein loading buffer. The immunoprecipitates and input were separated on a 10% SDS–PAGE gel and immunoblotted with anti-GST or anti-His antibodies. The primers used for constructing the related plasmids are listed in Supplementary Table S1.

Subcellular localization of the USB1 protein

The subcellular localization analysis was performed essentially as described previously (Mei et al., 2014). The coding region of the USB1 linked to GFP was cloned into the pROK219 vector, driven by the CaMV 35S promoter (35S::USB1-GFP). SOAR1 was used as a marker for cytosolic–nuclear localization (Mei et al., 2014) and the bHLH (basic helix-loop–helix) transcription factor FBI1/HFR1 was used as a nuclear–localized marker (Fairchild et al., 2000; Jung et al., 2005). The 35S::USB1-GFP plus SOAR1-RFP (red fluorescence protein) or 35S::USB1-GFP plus FBI1-RFP were transiently co-expressed in Arabidopsis protoplasts using the polyethylene glycol-mediated transformation protocol (Yoo et al., 2007). The fluorescence signal of GFP and RFP was detected using a confocal microscope (Zeiss LSM780, Germany). The 35S::USB1-GFP plasmid was also transformed into onion epidermal cells by particle bombardment-mediated transformation with a gene gun system (Bio-Rad). The samples were cultured at 26°C for 16 h, and then observed with a confocal laser scanning microscope (Zeiss, LSM780, Germany). In addition, the subcellular localization of USB1 was also assayed in the complementation lines (Com11-1) expressing 35S::USB1-GFP in the usb1-1 background. The fluorescence signals of GFP and DAPI (for nuclear staining) were imaged under a confocal laser scanning microscope (Zeiss LSM780, Germany). The primers for construction are listed in Supplementary Table S1.

qRT-PCR

Total RNA was extracted from 2-week-old plants with a Total RNA Rapid Extraction Kit (BioTeke, RP1202, China), treated with RNase-free DNase I (NEB, M0303S, USA) to degrade genomic DNA, and then purified using an RNA Purification Kit (BioTeke, RP1802, China) according to the manufacturer’s instructions. A 5 μg aliquot of RNA was used for first-strand cDNA synthesis using the Transcriptor cDNA synthesis Kit (Roche, 4 897 030 001, Germany). The primers to amplify spliced and unspliced mRNA are listed in Supplementary Table S1. All the experiments were repeated at least three times along with three independent repetitions of the biological experiments.

RNA sequencing (RNA-seq)

For the RNA-seq experiments, total RNA was extracted from 10-day-old plants, treated with RNase-free DNase I, and purified as described above. mRNA-seq libraries were constructed by following the standard Illumina protocol with three biological replicates per genotype. The Illumina sequencing was performed with an Illumina HiSeq 2500 paired-end system. For each sample, RNA-seq raw reads were trimmed using cutadapt v1.16 (Martin et al., 2011) to remove the potential Illumina adaptor contamination and conduct read trimming and clipping of the low quality bases. The remaining reads were aligned to the A. thaliana genome sequence and the reference-annotated genes (TAIR10) using STAR v2.5.3a (Dobin et al., 2013). Based on the RNA-seq mapped reads and the reference-annotated transcripts, featureCounts1.6.2 (Liao et al., 2014) was used to calculate the gene counts. rMATS (Shen et al., 2014) was used to detect differential alternative splicing events for our RNA-seq samples. Among the alternative spliced events, the absolute value of the Inc Level Difference>0.05 and P-value<0.05 showed significantly and differentially alternative splice events. R.mats2ashmutilpt was used to convert the alternative splicing analysis output into splicing visualization.

Measurement of intron retention and splicing efficiency

For assaying the intron retention and splicing efficiency, total RNA was extracted from 10-day-old plants, treated with RNase-free DNase I, and then purified as described above. A 5μg aliquot of RNA was used for first-strand cDNA synthesis with the Transcriptor cDNA synthesis Kit (Roche, 4 897 030 001, Germany). The relative unspliced mRNA level was tested by qRT-PCR using unspliced primers. The relative spliced mRNA level was tested by qRT-PCR using spliced primers. The splicing efficiency was determined by calculating the ratios of the level of spliced mRNA relative to the level of total mRNA (spliced plus unspliced mRNA). ACTIN2/8 genes were used as internal controls. The primers to amplify spliced and unspliced mRNA are listed in Supplementary Table S1. All the experiments were repeated at least three times along with three independent repetitions of the biological experiments.

Gene ontology analysis

The gene ontology (GO) analysis was conducted using the agrigo platform, which is a GO analysis toolkit specifically focused on agricultural species. The singular enrichment analysis (SEA) was chosen as the analysis tool and the suggested A. thaliana backgrounds were selected as the reference. The hypergeometric statistical test and Bonferroni multitest adjustment were used as the statistical test methods. The significance level threshold is 0.05; five was set as the minimum number of mapping entries (Tian et al., 2017).

Results

USB1 interacts with SOAR1 in the nucleus and cytoplasm

We previously showed that the PPR–domain protein SOAR1 plays a crucial role in ABA signaling (Mei et al., 2014; Jiang et al., 2014, 2015; Bi et al., 2019). In a yeast two–hybrid screen for SOAR1-interacting proteins, we found that Arabidopsis USB1, a homolog of USB1 in yeast and human (Supplementary Fig. S1), is a potential SOAR1–interaction partner. We confirmed the interaction between USB1 and SOAR1 with four independent experiments. In an in vitro pull-down assay, the SOAR1-His fusion protein was pulled down by anUSB1–GST fusion protein but not by the GST tag alone (a negative control) (Fig. 1A). In an in planta Co-IP assay with the total proteins extracted from two transgenic plants expressing GFP or USB1–GFP fusion protein, SOAR1 protein was co-immunoprecipitated by USB1–GFP but not by GFP (a negative control) (Fig. 1B). In the tobacco LCI assay, co-expression of SOAR1 plus USB1 in tobacco (N. benthamiana) leaves resulted in strong Luc fluorescence, whereas no Luc activity was detected in the negative controls (Fig. 1C). In the BiFC assay, the fluorescence signal appeared when SOAR1 plus USB1 were co-expressed in Arabidopsis protoplasts, but no signal was found with the negative controls (Fig. 1D). All these data consistently indicate that USB1 specifically interacts with SOAR1.
USB1 regulates ABA signaling

**Fig. 1.** USB1 co-localizes and physically interacts with SOAR1 in both the nucleus and cytoplasm. (A) *In vitro* pull-down assay to test the direct interaction between USB1 and SOAR1 protein. The SOAR1-His proteins were incubated with immobilized GST or USB1-GST proteins, and the antibody against GST tag (IP: anti-GST) was used to pull down the purified USB1-GST or GST protein. Both the immunoprecipitated fractions and input were tested by immunoblotting with anti-His and anti-GST antibodies, respectively. (B) Co-immunoprecipitation (Co-IP) assay to test the interaction of USB1 with SOAR1 in Arabidopsis. Total proteins, extracted from the homozygous transgenic plants expressing GFP or USB1–GFP fusion protein (USB1–OE3), were immunoprecipitated with anti-GFP antibody (IP: anti-GFP). The immunoprecipitates and the input were tested by immunoblotting with anti-SOAR1 and anti-GFP antibodies. (C) Luciferase complementation imaging (LCI) assay to test the interaction between USB1 and SOAR1 using the *N. benthamiana* system. The coding sequences of USB1 or SOAR1 were cloned into the N-terminal fragment of Luc (NLuc) and the C-terminal fragment of Luc (CLuc) to form USB1–NLuc (or SOAR1–NLuc) and CLuc–USB1 (or CLuc–SOAR1), respectively. The constructs pairs of USB1–NLuc/CLuc–SOAR1 and CLuc–USB1/SOAR1–NLuc were co-injected into *N. benthamiana* leaves, and the Luc signals were observed 72 h after infiltration. The combinations of USB1–NLuc/CLuc and SOAR1–NLuc/CLuc vectors were used as negative controls. (D) Bimolecular fluorescent complementation imaging (BiFC) assay using yellow fluorescent protein (YFP) to test the interaction between USB1 and SOAR1 in Arabidopsis mesophyll protoplasts. The coding region of USB1 or SOAR1 was fused to the N-terminus of YFP (USB1–NYFP or SOAR1–NYFP), or to the C-terminus of YFP (USB1–CYFP or SOAR1–CYFP). The constructs pairs (as indicated) were co-transformed into Arabidopsis wild-type (Col-0) mesophyll protoplasts. The combinations of NYFP/ SOAR1–CYFP or USB1–CYFP were used as negative controls. The YFP fluorescence was imaged under a confocal laser scanning microscope. YFP, YFP signal; Auto, chloroplast autofluorescence signal; Merged, merged image of the YFP signal with the chloroplast autofluorescence signal. (E) Transgenic expression of the USB1–GFP fusion protein in whole Arabidopsis plants, showing that the USB1–GFP fusion protein (left) is localized to both the nucleus and cytoplasm in the root of a transgenic line (Com11-1, see Supplementary Fig. S5) expressing 35S::USB1-GFP in the usb1-1 background. Note that the expression level of USB1 in the Com11-1 transgenic line is slightly higher than that in the wild-type plants (Supplementary Fig. S5). The nuclei are indicated by DAPI staining. Bright, bright field; Merged, merged image of the DAPI signal with the USB1–GFP signal in the bright field. (F) Bifurcation of the USB1–GFP fusion protein in the Arabidopsis protoplasts, showing that the nuclear–cytoplasmic dual localization of USB1. Left panels: the signal of the USB1–GFP fusion protein overlaps the signal of the nuclear-localized bHLH (basic helix-loop-helix) transcription factor FBI1/HFR1 (FBI1–RFP tagged with mCherry (a red fluorescent protein) in the nuclear portion (Merged, merged image of USB1–GFP with FBI1–RFP in the bright field). Right panels: the signal of the USB1–GFP fusion protein completely overlaps the signal of the cytosolic–nuclear dual-localized SOAR1–RFP (SOAR1 tagged with mCherry) fusion protein (Merged, merged image of USB1–GFP with SOAR1–RFP in the bright field). Bright, bright-field. (G) BiFC assays in the *N. benthamiana* leaves, indicating that USB1 interacts with SOAR1 in both the nucleus and cytoplasm. The coding regions of USB1 and SOAR1 were fused to the N-terminus of YFP and the C-terminus of YFP to form USB1–NYFP and SOAR1–CYFP, respectively. The construct pairs (as indicated) were co-infiltrated into *N. benthamiana* leaves (Merged, merged image of the YFP signal with the DAPI signal). All the experiments were repeated five times with similar results.

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**USB1 regulates ABA signaling**

USB1, a ubiquitin-domain-containing protein, has been shown to play a role in the regulation of ABA signaling. USB1 localizes to both the nucleus and cytoplasm, suggesting its dual role in the regulation of ABA signaling. The interaction between USB1 and SOAR1, a protein involved in ABA signaling, was confirmed using several assays, including pull-down assays, co-immunoprecipitation, and luciferase complementation imaging. The results indicate that USB1 and SOAR1 interact in both the nucleus and cytoplasm, suggesting a complex regulatory mechanism in ABA signaling. The interaction between USB1 and SOAR1 was further confirmed using bimolecular fluorescent complementation imaging, showing co-localization in both the nucleus and cytoplasm.

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*USB1 regulates ABA signaling*
We conducted transgenic expression of the USB1–GFP fusion protein in Arabidopsis T-DNA insertion knockout mutant plants (usb1-1; Fig. 2A, B), and selected a transgenic line (Com11-1; Supplementary Fig. S5) in which the expression level of USB1 is slightly higher than that of the wild-type plants to investigate USB1 subcellular localization. We observed that the USB1 protein is localized to both the nucleus and cytoplasm in root cells (Fig. 1E). The transient expression of the USB1–GFP fusion protein in both Arabidopsis protoplasts and onion epidermal cells verified the nuclear–cytoplasmic dual localization of USB1 (Fig. 1F; Supplementary Fig. S2A), and showed that USB1 co-localizes to the nucleus and cytoplasm with SOAR1 (Fig. 1F). We also showed that the USB1 gene is expressed in nearly all tissues/organs (Supplementary Fig. S2B), which is similar to the expression profile of the SOAR1 gene (Mei et al., 2014). Further, we performed BiFC assay in tobacco (N. benthamiana) leaves to examine the subcellular compartment in which the interaction of USB1 with SOAR1 takes place, and showed that USB1 localizes to and interacts with SOAR1 in both the nucleus and the cytoplasm (Fig. 1G). This is consistent with the nuclear–cytoplasmic co-localization of these two proteins (Fig. 1F).

Knockout of USB1 increases, but its overexpression reduces, ABA sensitivity in post-germination growth

We obtained a mutant usb1-1 line (SAIL_717_G03), which contains a T-DNA insertion in the first exon as a knockdown allele (Fig. 2A), as evidenced by qRT-PCR (Fig. 2B). Further, we selected a single guide RNA target C1 in the USB1 gene to generate the usb1-2 mutant with the CRISPR/Cas9 system (Z.P. Wang et al., 2015). The usb1-2 mutant has an adenosine insertion in the third exon of the USB1 gene locus, which generates a premature stop codon and thus would lead to truncated proteins due to a coding frame shift (Fig. 2C).

We investigated ABA sensitivity of the different genotypes in early seedling growth with the seeds sown either directly in ABA-containing medium or in ABA-free medium and then transferred to ABA-containing medium 60 h after stratification. With these two methods we consistently observed that the usb1-1 and usb1-2 mutants showed an ABA-hypersensitive phenotype (Fig. 2D, E; Supplementary Figs S3, S4), while the USB1 overexpression lines displayed an ABA-insensitive phenotype compared with wild-type Col-0 plants (Fig. 2F, G; Supplementary Figs S3, S4) in ABA-induced post-germination growth arrest. It is noteworthy that the early seedling growth of the usb1-1 and usb1-2 mutants was significantly reduced compared with wild-type Col-0 plants in the ABA-free medium (Fig. 2D, E; Supplementary Fig. S4), suggesting that these mutants are hypersensitive to physiological levels of endogenous ABA. Also, we estimated the effect of ABA on seedling growth with relative growth values based on the absolute values (Fig. 2; Supplementary Figs S3, S4, S8). The ABA-hypersensitive phenotype in the usb1-1 and usb1-2 mutants was rescued by introduction of the USB1-coding sequence into the usb1-1 and usb1-2 plants (Supplementary Fig. S5), demonstrating that the ABA-hypersensitive phenotype was caused by the disruption of the USB1 gene in these mutants. These data reveal that USB1 negatively regulates ABA signaling in early seedling growth.

Additionally, we observed that the two usb1 mutant alleles showed hypersensitive phenotypes to both NaCl and mannitol treatments, but the USB1 overexpression lines displayed insensitive phenotypes in early seedling growth (Supplementary Figs S6, S7). It is noteworthy that ABA hypersensitivity usually enhances tolerance to salt and osmotic stress-induced growth inhibition, whereas ABA insensitivity reduces it. However, in the early post-germination stage, we observed opposite phenotypes, that is, these mutants show similar sensitivities to salt and osmotic stresses as to ABA. This could be attributed to alteration of ABA sensitivity in these mutants under the salt- and osmotically induced high concentrations of ABA (Zhu, 2002; Shinozaki et al., 2003).
USB1 regulates ABA signaling in early seedling growth. (A–C) Identification of the usb1 mutants. (A) For the usb1-1 mutant (with Col-0 background; SAIL_717_G03 from ABRC), the T-DNA was inserted into the first exon of the At5G51170 genome locus at 19 bp downstream of the start codon (ATG). Boxes and lines represent exons and introns, respectively. The usb1-2 mutant was generated by CRISPR/Cas9 technology. The target site (C1) for CRISPR/Cas9 technology is shown. Protospacer adjacent motifs (PAMs) are marked with bold letters. (B) qRT-PCR analysis of the USB1 expression level in seedlings of the 2-week-old wild-type Col-0, usb1-1, and USB1 overexpression lines OE1 and OE3. ACTIN2/8 genes were used as internal controls. The expression level of USB1 in Col-0 was standardized as one unit. Each value is the mean ±SE of three independent biological determinations. (C) The mutation in usb1-2, which was evaluated by sequencing. An adenosine insertion of the usb1-2 mutant in the USB1 gene locus at 551 bp downstream of ATG is indicated by a red letter A and an arrow. (D–G) Early seedling growth of wild-type Col-0, usb1-1 and usb1-2 mutants (D, E, and Col-0, OE1 and OE3 USB1 overexpression lines (F, G). Seeds were directly planted in ABA-free MS medium (0 μM) or MS medium supplemented with different concentrations of (±)-ABA (0.2 μM and 0.4 μM in D and E, and 1.0 μM and 1.5 μM in F and G), and the seedling growth was investigated 10 d after stratification at 4 °C for 3 d. (E, G) Statistical analysis of root length (left) and relative root length (right) of different genotypes described in (D) and (F), respectively. Relative values of the root length of each genotype grown on MS medium containing 0.2 μM and 0.4 μM (D, E) or 1.0 μM and 1.5 μM (F, G) (±)-ABA were normalized relative to the value of the corresponding genotype at 0 μM ABA, which was taken as 100%. In (E, G), each value is the mean ±SE of five biological determinations, and different letters represent significant differences at P<0.05 (Duncan’s multiple range test) when comparing values within the same ABA concentration.
USB1 and SOAR1 function synergistically and are both required in ABA-induced post-germination growth arrest

We previously showed that knockout of SOAR1 in the sober1-2 and sober1-3 mutants results in ABA-hypersensitive phenotypes, and overexpression of SOAR1 almost completely abolishes ABA-induced inhibition of seed germination and post-germination growth arrest (Jiang et al., 2014, 2015; Mei et al., 2014). We therefore tested whether USB1 genetically interacts with SOAR1 in ABA signaling. We observed that the usb1-1 sober1-2 and usb1-2 sober1-2 double mutants displayed a stronger ABA-hypersensitive phenotype than their corresponding single mutantsusb1-1, usb1-2, and sober1-2 (Fig. 3A; Supplementary Fig. S8A, B), indicating that USB1 functions synergistically with SOAR1 in ABA signaling.

Further, we observed that introduction of the usb1-1 or usb1-2 mutant allele into the SOAR overexpression line OE6 (SOAR1-OE6) partly suppressed the strong ABA-insensitive phenotype of SOAR1-OE6 in early seedling growth (Fig. 3B; Supplementary Fig. S8C, D), revealing that the function of SOAR1 in ABA signaling requires USB1. Similarly, we showed that introduction of the sober1-2 mutation into the USB1 overexpression line OE3 (USB1-OE3) partly suppressed the ABA-insensitive phenotype of USB1-OE3 in early seedling growth (Fig. 3C), indicating that the role of USB1 in ABA signaling also depends on SOAR1. Taken together, these data reveal that USB1 and SOAR1 function interdependently and cooperatively in ABA-induced post-germination growth arrest.

Knockout of USB1 or SOAR1 results in a wide range of pre-mRNA splicing defects, and double mutation of the two genes increases the defects

We performed high-throughput RNA-seq analysis to clarify the expression profiles regulated by USB1 and SOAR1. The data revealed that the expression levels of 1372 genes (833 + 539) in usb1-1, and 3409 genes (2870 + 539) in sober1-2 were changed (|fold change| > 1.5, P < 0.05) in comparison with wild-type plants without ABA treatment, where expression of 539 genes was potentially co-regulated by USB1 and SOAR1 (Fig. 4A). ABA treatment enhanced the number of genes (1746 genes in usb1-1, 3591 genes in sober1-2) whose expression levels were altered, with 593 genes potentially co-regulated by USB1 and SOAR1 (Fig. 4A). It is noteworthy that USB1 and SOAR1 oppositely affected a set of genes, and notably cooperated to up- or down-regulate their expression levels. Moreover, ABA treatment increased the number of down-regulated genes (Fig. 4B).

We further analyzed the differential alternative splicing events from RNA-seq data, and found 576 alternative splicing events for 526 genes in the usb1-1 mutant, while 359 alternative splicing events were found for 334 genes in sober1-2 compared with wild-type plants without ABA treatment. ABA treatment probably reduced alternative splicing events, with 466 alternative splicing events in 426 genes in usb1-1, and 353 alternative splicing events in 326 genes in sober1-2 (Fig. 4C; Supplementary Fig. S9). It is particularly noteworthy that the total number of splicing events and involved genes increased in the usb1-1 sober1-2 double mutant compared with that in the usb1-1 or sober1-2 single mutant (Fig. 4C; Supplementary Fig. S9). Additionally, we observed that intron retention is the most abundant alternative splicing category amongst the five categories of alternative splicing events: intron retention, exon skipping, alternative 5' SS, alternative 3' SS, and mutually exclusive exon (Supplementary Fig. S9).

GO analysis of the alternative splicing genes, which are regulated by USB1 (Fig. 4D) and co-regulated by both USB1 and SOAR1 (Fig. 4E; Supplementary Fig. S10; Supplementary Table S2), revealed that these genes were involved in many biological processes, such as the response to abiotic stimulus, light stimulus, hormone responses, developmental process, and primary metabolic processes. This implies that USB1 and SOAR1 cooperate to modulate a set of biological processes by regulating pre-mRNA splicing.

Knockdown of USB1 or SOAR1 affects accurate mRNA splicing of a subset of ABA-responsive genes, and double mutation of the two genes enhances the defects

RNA-seq analysis showed the intron retention of several ABA-responsive genes in usb1-1 and sober1-2 mutants, such as HAB1.1, CIPK3, MYB9, and AT1G14170. HAB1 (At1g72770) encodes a member of the PP2Cs negatively involved in ABA signaling (Umezawa et al., 2009; Vlad et al., 2009). HAB1 transcripts have four variants, HAB1.1, HAB1.2, HAB1.3, and HAB1.4, in which retention of the third intron (unspliced) mRNA transcript was regarded as HAB1.2 and spliced mRNA as HAB1.1 (Z. Wang et al., 2015; Zhan et al., 2015). We showed that the levels of HAB1.1 and HAB1.2 were altered in the usb1-1 and sober1-2 single and usb1-1 sober1-2 double mutants compared with wild-type plants (Fig. 5A; Supplementary Fig. S11). This results in a significantly increased ratio of HAB1.2 to HAB1.1 in these mutants, especially in the usb1-1 sober1-2 double mutant in comparison with wild-type plants, and ABA treatment amplified the differences in the ratio of HAB1.2 to HAB1.1 among these genotypes (Fig. 5A; Supplementary Fig. S11).

The CIPK3 gene (At2g26980) encodes a calcineurin B-like-interacting protein kinase 3 that negatively regulates ABA signaling (Kim et al., 2003; Pandey et al., 2008; Sanjaly et al., 2017a, b). MYB9 (At5g16770) encodes an ABA-responsive transcription factor (Chen et al., 2006). The At1g14170 locus encodes an RNA-binding protein, whose phosphorylation level is repressed by ABA (Kline et al., 2010). We showed that the levels of alternative splicing events for these genes were significantly altered in the usb1-1 and sober1-2 single and usb1-1 sober1-2 double mutants compared with wild-type plants, particularly with the most marked change in the usb1-1 sober1-2 double mutant, which results in a significant decrease of the mature mRNA levels (relative spliced mRNA levels) and splicing efficiency (Fig. 5B–D; Supplementary Fig. S12). It is noteworthy that ABA treatment significantly affected these
Given that ABI5 was previously shown to be a downstream player of ABAR–SOAR1 coupled signaling (Mei et al., 2014) and that the ABI5 mRNA is a target of the mRNA cap-binding complex in SOAR1–eIFiso4G1/2 coupled signaling,

Fig. 3. USB1 functionally interacts with SOAR1 in ABA-induced early seedling growth inhibition. (A) Seedling growth of the wild-type Col-0, usb1-1 and soar1-2 single mutants, and the usb1-1 soar1-2 double mutant in ABA-free (0 μM) and ABA-containing (0.2 μM) medium 10 d after stratification at 4 °C for 3 d. (B) Seedling growth of wild-type Col-0, usb1-1, the SOAR1 overexpression line OE6 (SOAR1-OE), and SOAR1-OE under the usb1-1 background (usb1-1 SOAR1-OE) in ABA-free (0 μM) and ABA-containing (10μM) medium. (C) Seedling growth of wild-type Col-0, soar1-2, and USB1-OE3 under the soar1-2 background (soar1-2 USB1-OE3) in ABA-free (0 μM) and ABA-containing (1.0 μM) medium. In (A–C), the histograms (indicated by red arrows) show statistical analysis of the relative root length of the corresponding different genotypes, and the relative root length of each genotype grown on ABA-containing MS medium was normalized relative to the value of the corresponding genotype at 0 μM ABA, which was taken as 100%. Each value is the mean ±SE of five biological determinations, and different letters indicate significant differences at P<0.05 (Duncan’s multiple range test) when comparing values within the same ABA concentration.

splicing events compared with control treatment (Figs. 5B–D). Taken together, these findings indicate that both USB1 and SOAR1 are required for the accurate mRNA splicing of the ABA-responsive genes.
we tested if ABI5 expression is altered in the usb1-1 and usb1-2 mutants. We found that loss-of-function of USB1 did not affect ABI5 expression (Supplementary Fig. S13), suggesting that ABI5 may not be a target of USB1 in this pre-mRNA splicing event.

Previous studies in Arabidopsis and maize showed that U6 snRNP plays a crucial role in plant development throughout the life cycle. The Arabidopsis LSM2–LSM8 complex is an essential component of the U6 snRNP and LSM1- and LSM8-deficient mutants display severe developmental alterations (Perea-Resa et al., 2012). Loss-of-function mutation of the maize USB1 gene affects seed development (Li et al., 2017). Given that Arabidopsis USB1 and SOAR1 cooperate to regulate a wide range of pre-mRNA splicing involved in diverse developmental processes as mentioned above (Fig. 4; Supplementary Figs. S9, S10; Supplementary Table S2), we investigated physiological consequences caused by loss-of-function or overexpression of USB1 during the life cycle of these plants. We observed that loss-of-function of USB1 reduced, but overexpression of USB1 enhanced, plant growth, as estimated by both height and weight, silique length and number, and seed size and total weight (Supplementary Fig. S13). Thus, loss-of-function of USB1 severely affects, but overexpression of USB1 improves plant growth and development, suggesting the potential use of the USB1 gene in crop improvement. These findings, consistent with previous observations (Perea-Resa et al., 2012), indicate the crucial roles of U6 snRNP in the whole developmental process.
Fig. 5. USB1 and SOAR1 cooperate to regulate the alternative splicing of a subset of genes involved in ABA/stress signaling. (A) Top: diagrams of the HAB1.1 (also called spliced mRNA) and HAB1.2 (also called unspliced mRNA) variants. Boxes and lines represent exons and introns, respectively (figure drawn to scale). E1–E4 represent exons 1–4. Bottom left panel: the intron retention events of HAB1, visualized by Rmats2sashimiplot in the wild-type Col-0, usb1-1 and soar1-2 single mutants, and usb1-1 soar1-2 double mutant without ABA treatment. Bottom right panel: the HAB1 variants in the different genotypes as described in the left panel, identified by qRT-PCR under ABA-free (–ABA) or ABA treatment (+ABA). Primers for detecting HAB1.1 (spliced mRNA) and HAB1.2 (unspliced mRNA) transcripts are shown in the diagram in the left panel, in which the dashed line indicates the intron, and the black/bold line the exon. UF represents the unspliced forward primer and UR the unspliced reverse primer for detecting the unspliced mRNA level, which are designed according to the unspliced intron and adjacent exon, respectively (Supplementary Table S1). SF, spliced forward primer, and SR, spliced reverse primer for detecting the spliced mRNA level, are designed according to the sequences across exon–exon junctions, in which the reverse primer SR is the same as UR. (B–D) The intron retention events of CIPK3 (retention of intron 12, B), MYB9 (retention of intron 1, C), and AT1G14170 (retention of intron 1, D), visualized by the Rmats2sashimiplot (left, color panels) and identified by qRT-PCR under ABA-free (–ABA) or ABA treatment (+ABA) (right, column panels) in Col-0, usb1-1 and soar1-2 single mutants, and the usb1-1 soar1-2 double mutant. The primers UF, UR, SF, and SR for detecting spliced and unspliced mRNA transcripts, shown in the corresponding diagram, are the same as described in (A). Splicing efficiency of each genotype (right column) is referred to as the ratio of the level of spliced mRNA relative to the level of total mRNA (spliced plus unspliced mRNA). Each value is the mean ±SE of five biological determinations, and different letters indicate significant differences at $P<0.05$ (Duncan’s multiple range test) when comparing values of the different genotypes within the same treatment (–ABA, ABA-free; and +ABA, ABA treatment).
Discussion

USB1 directly interacts with SOAR1 to negatively regulate ABA signaling

As a 3′–5′ exoribonuclease that potentially generates U6 snRNA for mRNA spliceosome assembly, USB1 was shown to regulate seed development in maize (Li et al., 2017), but the function of Arabidopsis USB1 remained unknown. In the present study, we showed that USB1 is an interaction partner of SOAR1 (Fig. 1). Knockout of the USB1 gene increases ABA sensitivity in early seedling growth (Fig 2) but overexpression reduces it, demonstrating that USB1 functions negatively in ABA signaling. This negative role of USB1 is consistent with that of its interaction partner SOAR1, which functions as a crucial, negative player of ABA signaling (Jiang et al., 2014, 2015; Mei et al., 2014). Further, we showed that the usb1-1 soar1-2 and usb1-2 soar1-2 double mutants displayed additive ABA-hypersensitive phenotypes compared with either single mutant alone (Fig. 3), indicating that USB1 functions synergistically with SOAR1 in ABA signaling. This suggests that USB1 functions in either an independent/parallel pathway from SOAR1 or in the same pathway. It is particularly noteworthy that the soar1 null allele is lethal and soar1-2 is a knockdown allele (Mei et al., 2014). The additive ABA-hypersensitive phenotypes of the usb1 soar1-2 double mutants suggest that USB1 and SOAR1 may function in the same pathway rather than in a parallel pathway, given that adding together two hypomorphic loss-of-function mutations in genes within the same pathway would be expected to lead to additive phenotypes. Further genetic findings revealed that USB1 and SOAR1 are mutually required to function cooperatively in ABA-induced post-germination growth arrest (Fig. 3), supporting the idea that USB1 functions in the same pathway as SOAR1 and at the same signaling node.

USB1 and SOAR1 cooperatively regulate pre-mRNA splicing involved in ABA signaling

Alternative pre-mRNA splicing was reported to be involved in plant responses to ABA, high salinity, extreme temperatures, and drought stress (Xiong et al., 2001; Zhang et al., 2011; Cui et al., 2014; Kong et al., 2014; Z. Wang et al., 2015; Zhan et al., 2015; Carrasco-Lopez et al., 2017; Kim et al., 2017). However, the mechanism by which ABA signaling affects the alternative splicing process remains largely unknown. In the present study, we showed a potential mechanism involving both USB1 and SOAR1. Previous reports showed that USB1 is involved in the pre-mRNA splicing events in yeast and maize, where loss-of-function of the USB1 gene leads to pre-mRNA splicing defects (Shchepachev et al., 2012; Li et al., 2017). Consistent with these previous findings, we observed that underexpression of USB1 or SOAR1 results in a wide range of pre-mRNA splicing defects in which the mRNA splicing of a subset of ABA-responsive genes was affected, and double mutation of USB1 and SOAR1 genes enhanced the defects (Figs 4, 5; Supplementary FigsS9–12; Supplementary Table S2). These findings indicate that both USB1 and SOAR1 are required for accurate mRNA splicing of ABA-responsive genes.

It is particularly noteworthy that underexpression of USB1 or SOAR1 alters pre-mRNA splicing of a member of the clade-A PP2Cs, which negatively regulate ABA signaling by dephosphorylating SnRK2 protein kinases, especially SnRK2.6 (Umezawa et al., 2009; Vlad et al., 2009). The HAB1 pre-mRNA undergoes alternative splicing to produce two functional splice variants HAB1.1 and HAB1.2. HAB1.2 is shorter than HAB1.1 because of retention of the third intron generating a premature stop codon. HAB1.2 protein can interact with SnRK2.6 but fails to dephosphorylate OST1 kinase activity, probably by which HAB1.2 positively regulates ABA signaling. Importantly, the ratio of HAB1.2 to HAB1.1 alternative splice forms was shown to be an on and off switch in ABA signal transduction (Z. Wang et al., 2015; Zhan et al., 2015). We showed that knockout of USB1 or SOAR1 in the usb1-1 and soar1-2 single mutants increased the ratio of HAB1.2 to HAB1.1, with amplified effects in the usb1-1 soar1-2 double mutant, especially after ABA treatment (Fig. 5A; Supplementary Fig. S11). This suggests that USB1- and SOAR1-coupled signaling, together with the previously reported function of a homolog of human splicing factor RBM25, ROA1/RBM25 (Z. Wang et al., 2015; Zhan et al., 2015), regulates alternative splicing of HAB1 to participate in ABA signaling. The higher ratio of HAB1.2 to HAB1.1 explains, at least partly, the ABA-hypersensitive phenotype of usb1-1 and soar1-2 single and usb1-2 soar1-2 double mutants (Figs 2, 3; Supplementary Figs S3–8).

Previous reports showed that the CIPK3 transcript has five splice variants, namely CIPK3.1, CIPK3.2, CIPK3.3, CIPK3.4, and CIPK3.5, among which only CIPK3.4 is a functional transcript accurately spliced with all 14 introns deleted (Sanyal et al., 2017). We found that the splicing efficiency of CIPK3 and the level of the mature CIPK3 mRNA increased in the usb1-1 and usb1-1 soar1-2 mutants (Fig. 5B; Supplementary Fig. S12), indicating that USB1/SOAR1-coupled signaling positively regulates splicing of functional CIPK3 mRNA coding for a negative regulator of ABA signaling (Kim et al., 2003; Pandey et al., 2008). This is consistent with the ABA-hypersensitive phenotype in usb1-1 single and usb1-1 soar1-2 double mutants (Figs 2, 3; Supplementary Figs S3–8). USB1 and SOAR1 also regulated mRNA splicing of the ABA-responsive transcription factor MYB9 (Chen et al., 2006) and the ABA-responsive RNA-binding protein encoded by the At1g14170 locus (Kline et al., 2010) (Fig. 5; Supplementary Fig. S12), suggesting that USB1 and SOAR1 also play important regulatory roles for these modulators of ABA signaling.

Previous studies showed that the essential components of the Arabidopsis U6 snRNP, LSM4/5/8, regulate pre-mRNA splicing of stress-responsive genes to modulate plant responses to environmental stresses (Zhang et al., 2011; Cui et al., 2014; Carrasco-Lopez et al., 2017). Importantly, Carrasco-Lopez et al. (2017) revealed that the Arabidopsis LSM2–LSM8 complex regulates spliceosome activity specifically in response to changing environmental conditions, ensuring the efficiency and accuracy of constitutive and alternative splicing of selected pre-mRNAs, and thus adequate plant adaptation to abiotic stresses (Carrasco-Lopez et al., 2017). USB1- and SOAR1-coupled
ABA signaling may function in a similar manner, ensuring an adequate ABA response to balance plant development and stress tolerance, but the regulatory details of this process will require further research.

We previously reported that SOAR1 is a unique cytoplasmic–nuclear dual-localized PPR protein negatively involved in ABA signaling (Mei et al., 2014; Jiang et al., 2014, 2015) partly by affecting assembly of the mRNA cap-binding complex to affect mRNA translation (Bi et al., 2019). The present study reveals that SOAR1 interacts with USB1 to regulate ABA signaling probably by regulating assembly of the mRNA spliceosome to modulate pre-mRNA splicing of ABA-responsive genes. These findings provide a new link of ABA signaling with the pre-mRNA splicing process, and help to understand complex ABA signaling pathways.

Supplementary data

The following supplementary data are available at JXB online.

Fig. S1. Phylogenetic analysis of Arabidopsis USB1 protein homologs.

Fig. S2. Expression profile of the USB1 gene and subcellular localization of USB1 protein.

Fig. S3. Early seedling growth of the usb1 mutants under higher concentrations of ABA.

Fig. S4. Early seedling growth of the usb1 mutants and USB1 overexpression lines, assayed by transferring germinating seeds to ABA-containing medium.

Fig. S5. Phenotypic analysis of the complementation lines of the usb1 mutants.

Fig. S6. Early seedling growth of different genotypes under salt stress.

Fig. S7. Early seedling growth of different genotypes under n-mannitol-induced osmotic stress.

Fig. S8. USB1 functionally interacts with SOAR1 in ABA-induced early seedling growth inhibition.

Fig. S9. Quantification of alternative splicing events.

Fig. S10. Gene Ontology analysis of genes co-regulated by USB1 and SOAR1 under mock and ABA treatment conditions.

Fig. S11. The intron retention events of HAB1 in the different genotypes.

Fig. S12. Diagrams of the intron retention events of CIPK3, MYB9, and AT1G14170 in the different genotypes under mock and ABA treatment conditions.

Fig. S13. Early seedling growth of the usb1 mutants and USB1 overexpression lines during the plants’ life cycle.

Table S1. Primers used in this study.

Table S2. RNA-seq data: function of genes co-regulated by USB1 and SOAR1.

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