Arabidopsis pentatricopeptide repeat protein SOAR1 plays a critical role in abscisic acid signalling

Chao Mei*, Shang-Chuan Jiang*, Yan-Fen Lu, Fu-Qing Wu, Yong-Tao Yu, Shan Liang, Xiu-Jing Feng, Sergi Portoles Comeras, Kai Lu, Zhen Wu, Xiao-Fang Wang† and Da-Peng Zhang†

MOE Systems Biology and Bioinformatics Laboratory, Center for Plant Biology, School of Life Sciences, Tsinghua University, Beijing 100084, China

* These authors contributed equally to this work.
† To whom correspondence should be addressed. E-mail: zhangdp@tsinghua.edu.cn or wangxf@biomed.tsinghua.edu.cn

Received 3 May 2014; Revised 6 June 2014; Accepted 9 June 2014

Abstract

A dominant suppressor of the ABAR overexpressor, soar1-1D, from CHLH/ABAR [coding for Mg-chelatase H subunit/putative abscisic acid (ABA) receptor (ABAR)] overexpression lines was screened to explore the mechanism of the ABAR-mediated ABA signalling. The SOAR1 gene encodes a pentatricopeptide repeat (PPR) protein which localizes to both the cytosol and nucleus. Down-regulation of SOAR1 strongly enhances, but up-regulation of SOAR1 almost completely impair, ABA responses, revealing that SOAR1 is a critical, negative, regulator of ABA signalling. Further genetic evidence supports that SOAR1 functions downstream of ABAR and probably upstream of an ABA-responsive transcription factor ABI5. Changes in the SOAR1 expression alter expression of a subset of ABA-responsive genes including ABI5. These findings provide important information to elucidate further the functional mechanism of PPR proteins and the complicated ABA signalling network.

Key words: Abscisic acid signalling, Arabidopsis thaliana, Mg-chelatase H subunit, pentatricopeptide repeat (PPR) protein, post-germination growth, seed germination.

Introduction

Pentatricopeptide repeat (PPR) proteins are a class of RNA-binding proteins characterized by the presence of a degenerate 35 amino acid repeat, the PPR motif, which is repeated in tandem 2–50 times. The PPR motifs form a helical structure and are considered to be RNA-binding motifs (Aubourg et al., 2000; Small and Peeters, 2000; Lurin et al., 2004). The first PPR gene was identified in Saccharomyces cerevisiae (Manthey and McKewen, 1995), and it is known that all sequenced eukaryotic genomes have been found to encode PPR proteins, though the numbers of PPR genes in both animal and fungal genomes are relatively small. The PPR domain protein family is particularly large in land plants. In the Arabidopsis thaliana genome, 450 putative PPR genes were identified, and >600 PPR genes have been predicted to occur in the rice genome (Small and Peeters, 2000; Lurin et al., 2004; Rivals et al., 2006; Schmitz-Linneweber and Small, 2008).

It has been known that PPR proteins are mostly targeted to mitochondria and/or chloroplasts in plants, and they are involved in many aspects of RNA processing in these two organelles, such as RNA splicing, editing, 5’ and 3’ end processing, stability and cleavage, and translation (Meierhoff et al., 2003; Williams and Barkan, 2003; Lurin et al., 2004). The mitochondrial/chloroplast PPR proteins play diverse and crucial roles in plant developmental processes and responses to environmental stresses (Small and Peeters, 2000; Lurin et al., 2004; Oguchi et al., 2004; Tzafrir et al., 2004; Cushing et al., 2005; Ding et al., 2006; Wang et al., 2006; Gutierrez-Marcos et al., 2007; Koussevitzky et al., 2007; Chi et al., 2008; Fujii and Small, 2011; Hu et al., 2012; Nakamura et al., 2012).
Few PPR proteins, however, have been found to localize to cellular compartments other than mitochondria and chloroplasts. To the authors’ knowledge, thus far, two PPR proteins have been identified as localizing to the nucleus, of which one PPR protein was found only in the nucleus and another in both the mitochondrion and the nucleus, and they regulate embryogenesis probably by modulating nuclear gene transcription and RNA processing (Ding et al., 2006; Hammani et al., 2011).

The phytohormone abscisic acid (ABA) regulates many developmental processes and plant adaptation to adverse conditions (reviewed in Finkelstein et al., 2002; Adie et al., 2007; Cutler et al., 2010). Numerous ABA signalling components, including receptors or candidate receptors for ABA, have been identified (Finkelstein et al., 2002, Gao et al., 2007; Johnston et al., 2007; Liu et al., 2007a, b; Guo et al., 2008; Ma et al., 2009; Pandey et al., 2009; Park et al., 2009; Santiago et al., 2009; Cutler et al., 2010). The START-domain family proteins PYR/PYL/RCAR are the best characterized cytosolic ABA receptors, which mediate a core ABA signalling pathway involving the downstream components such as the type 2C protein phosphatases (PP2Cs), SNF1-related protein kinase 2s (SnRK2s), and a clade of bZIP-domain transcription factors (AbA-INSENSITIVE5 (ABI5)). These data demonstrate that CHLH/ABAR is an essential ABA signalling regulator in plant cells.

To explore further the mechanism of the CHLH/ABAR-mediated ABA signalling, a suppressor of the ABA overexpression (named soar1-ID), in which the expression levels of both the CHLH/ABAR gene and the SOAR1 gene encoding a PPR-motif protein, are up-regulated, was screened. It was shown that SOAR1 localizes to both the cytosol and nucleus, and functions as a critical, negative, regulator of the ABA signalling pathway in seed germination and seedling growth. Genetic evidence revealed that SOAR1 acts downstream of ABAR and probably upstream of a nuclear ABA-responsive bZIP transcription factor ABA-INSENSITIVE5 (ABI5). These findings provide important information to elucidate further the functional mechanism of PPR proteins and the highly complicated ABA signalling network.

Materials and methods

Plant materials and growth conditions

ABA overexpression lines were generated by introducing an ABA gene (At5g13630) fragment (encoding a truncated ABAR with amino acid residues 631–999) into A. thaliana ecotype Columbia-0 (Col) plants as a green fluorescent protein (GFP) fusion protein. It was previously shown that the N-terminally truncated ABAR tagged with GFP functions similarly to full-length ABAR in transgenic plants, leading to ABA hypersensitivity in the major ABA responses (Ma et al., 2009). Therefore, the truncated ABAR overexpression lines were used as ABAR overexpressors. The cDNA isolation and transgenic manipulation were as previously described (Ma et al., 2009). From the population of the ABAR overexpression transgenic lines, the lines with ABA-insensitive or wild-type phenotypes in seed germination and post-germination growth were screened, which gave candidate suppressors of the ABAR overexpression (named soar mutant) lines. The soar1-ID mutant was identified from these candidate soar mutant lines.

The soar1-2 and soar1-3 (stock nos FLAG_546D07 and FLAG_500B04, respectively, with the Col ecotype as background) were obtained from Versailles Genetics and Plant Breeding Laboratory, Arabidopsis thaliana Resource Center (INRA; http://dbsgap.versailles.inra.fr/portail/). The seed of the abi5-1 (stock no. CS105, with the Wassilewskija ecotype as background; locus of the ABI5 gene, At1g36270) mutant was obtained from the Arabidopsis Biological Resource Center (ABRC; http://abrc.osu.edu/), and the background of the abi5-1 mutant was changed to the Col ecotype by crossing as described previously (Shang et al., 2010). The soar1-2 abi5-1 double mutant was created by crossing. The seeds of ab1-3 (stock no. SALK_076309) and abi2-2 (stock no. SALK_015166) mutants were also obtained from the ABRC, and the mutants are T-DNA insertion knockout alleles in the ABI1 (At4g26080) and ABI2 (At5g57050) genes, respectively. The background of both mutants is the Col ecotype. The ab1-3 abi2-2 double mutant is a generous gift from Y. Guo’s laboratory (College of Biological Sciences,
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China Agricultural University). All the primers for identification of the above-mentioned mutants are listed in Supplementary Table S1 available at JXB online.

The A. thaliana ecotype Col was used to generate transgenic plants. To generate the SOAR1 (At5g11310) overexpression lines, the full-length SOAR1 cDNA, amplified by PCR with the primers listed in Supplementary Table S1 at JXB online, was cloned into the binary vector pCAMBIA1300 (http://www.cambia.org), which contains the Cauliflower mosaic virus (CaMV) 35S promoter and the C-terminal GFP flag. Also, an ABR and SOAR1 double gene overexpression line was created by crossing an ABR overexpressor with a SOAR1 overexpressor (OE1) to test genetic interaction of these two genes. A previously generated ABI2 overexpression line (Sun et al., 2011; the ABI2-2 line harbouring GFP-tagged full-length ABI2 under the Col background) was used as a control in phenotypic analysis of the SOAR1 overexpression lines, which showed strong ABA-insensitive phenotypes (Sun et al., 2011) and was renamed ABI2-OE in the present study. To generate the transgenic complementation lines of the soar1-2 and soar1-3 mutants, the native promoter, isolated by PCR with the primers listed in Supplementary Table S1 was used, to replace the CaMV 35S promoter in the above-mentioned constructs to create the native promoter-driven SOAR1 construct. These constructs were introduced, respectively, into Agrobacterium tumefaciens strain GV3101 and transformed into the wild-type plants (for the SOAR1–overexpression lines) or soar1 mutants (for the complementation lines of the soar1-2 and soar1-3 mutants) by the floral dip infiltration method (Clough and Bent, 1998). The homozygous T3 seeds of the transgenic plants were used for analysis.

Plants were grown on Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) containing 3% (w/v) sucrose and 0.8% agar or in compost soil under a 16 h photoperiod in a growth chamber at ~20 °C. Plants were grown under a 12 h photoperiod at ~20 °C for protoplast preparation.

SOAR1 promoter–GUS transformation assay

The promoter of SOAR1 (pSOAR1) was amplified by PCR using forward primer 5′-AATCTGAGTCTGGCGAGAAAATTACAGAAACACATATAATGGAAGACAGA-3′ and reverse primer 5′-CGGGATCCTCCGCCGAGAAAATTACAGAAACACATATAATGGGAAGACAGA-3′. The PCR product was digested and cloned into the pCAMBIA1391 vector. The construct pSOAR1-GUS (β-glucuronidase) was transformed into Arabidopsis Col plants by floral infiltration. Histochemical staining was performed, as described previously (Jefferson et al., 1987), by soaking whole plants or tissues in a solution consisting of 1 mM X-gluc, 100 mM sodium phosphate buffer (pH 7.0), 0.05 mM K3Fe(CN)6, 0.05 mM K4Fe(CN)6, 2 mM EDTA, and 0.1% (v/v) Triton X-100 at 37 °C for 5–8 h. After GUS staining, chlorophyll was cleared from the tissues with a mixture of 30% acetic acid and 70% ethanol, and then the samples were investigated under a stereomicroscope (Olympus).

Quantitative real-time PCR and TAIL-PCR

Quantitative real-time PCR for mRNA expression levels of various genes (see Supplementary Table S1 at JXB online for the gene-specific primers) was performed as previously described (Shang et al., 2010) essentially according to the instructions provided for the Bio-Rad Real-Time System CFX96TM C1000 thermal cycler (Bio-Rad, Hercules, CA, USA). The T-DNA flanking sequence in the soar1-1 dominant mutant was determined by TAIL-PCR (thermal asymmetric interlaced PCR) with pCAMBIA1300-specific left border and random primers that are listed in Supplementary Table S1. The reaction program for rounds was described previously (Liu et al., 1995).

Seeds were stratified for 3 d at 4 °C, incubated on filter paper imbibed with ABA-free or ABA-containing solution for 24 h in a light growth chamber at 20 °C, and collected for RNA extraction. Total RNA was isolated from these germinating seeds with the RNasy plant mini kit (Qiagen) supplemented with an on-column DNA digestion (Qiagen R Nase-Free DNase set) according to the manufacturer’s instructions, and then the RNA sample was reverse-transcribed with the Superscript II RT kit (Invitrogen) in a 25 μl volume at 42 °C for 1 h. Amplification of ACTIN2/8 genes was used as an internal control. The cDNA was amplified using SYBR Premix Ex Taq (TaKaRa) with DNA Engine Opticon 2 thermal cycler in a 10 μl volume. The Ct (threshold cycle), defined as the PCR cycle at which a statistically significant increase of reporter fluorescence was detected, was used as a measure for the starting copy numbers of the target gene. Relative quantitation of the target gene expression level was performed using the comparative Ct method.

Arabidopsis protoplast and onion epidermis transformation

Transient transgenic manipulation in both Arabidopsis protoplasts and onion epidermis was used to assay the subcellular localization of the SOAR1 protein essentially as described previously (Shang et al., 2010). The full-length SOAR1 and a fragment of 106–1809 bp downstream of the transcription start site of SOAR1 (SOAR1106–1809), which encodes a truncated SOAR1 from amino acid residue 36 to 603 with the N-terminal 35 amino acid residues deleted) were amplified by PCR, and the products were cloned into the p-EASY-T1 vector (Transgen, Beijing, China) for sequencing, and then fused with GFP and inserted into the pROK219 vector, driven by the CaMV 35S promoter. The positive control cytosolic-nuclear marker PYRI (At4g17780; Park et al., 2009) and nuclear marker FBII1 (At1g02340; Lee et al., 2002) were used as described previously (Zhao et al., 2011), and their cDNAs were amplified and fused with mCherry (Shaner et al., 2004) in-frame into the pROK219 vector, driven by the CaMV 35S promoter, respectively. The primers for cloning the full-length SOAR1, SOAR1106–1809, PYRI, and FBII1 are listed in Supplementary Table S1 at JXB online. Protoplasts were transiently transformed using the polyethylene glycol-mediated transformation protocol (Yoo et al., 2007). The onion epidermal cells were transformed by particle bombardment-mediated transformation with the biolistic PDS-1000/HE gene gun system (Bio-Rad). Bombarded samples were cultured at 26 °C for 16 h, and then observed with a confocal laser scanning microscope (ZEISS, Oberkochen, Germany).

Isolation of cytosolic and nuclear fractions

The cytosolic and nuclear fractions were isolated essentially according to the protocol described previously (Shang et al., 2010). Ten-day-old Arabidopsis seedlings were ground to fine powder using liquid nitrogen and pre-chilled using a mortar and pestle. Cytosolic protein isolation buffer is composed of 10 mM HEPES, pH 8.0, 250 mM sucrose, 0.5% (v/v) Triton X-100, 1 mM EDTA, 5 mM MgCl2, 50 mM NaCl, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 1× Roche Cocktail (protease inhibitor cocktail). The buffer was added at 1 ml g–1 powder to generate the homogenate. After centrifuging at 10 000 g for 15 min, the supernatant was mixed with 2× SDS sample buffer and denatured for 10 min in boiling water. The isolated cytosolic fraction was examined by immunodetecting the presence of the nuclear marker histone H3 with anti-histone H3 antibody (Sigma-Aldrich) to verify that the cytosolic fraction was not contaminated by the nuclear fraction. The nuclear fraction was isolated according to the protocol of Cold Spring Harbor Laboratory as described on its website, and examined by immunodetecting the presence of the cytosolic marker PEPC (phosphoenolpyruvate carboxylase) with anti-PEPC antibody (Agrisera) to ensure that the nuclear fraction was not contaminated by the cytosolic fraction.

Antiserum production, protein extraction, and immunoblotting

The antisera against ABR and SOAR1 were produced and tested for specificity essentially with the same procedures as described previously (Wu et al., 2009; Shang et al., 2010). A truncated SOAR1 (303 amino acid residues from 299 to 602) was used as antigen for
production of the anti-SOAR1 serum. The primers for cloning the cDNA fragment to produce the truncated SOAR1 in Escherichia coli are listed in Supplementary Table S1 at JXB online. The produced anti-SOAR1 serum was tested and shown to be specific for SOAR1 protein (Supplementary Fig. S3). The extraction of the Arabidopsis total protein, SDS-PAGE, and immunoblotting were done essentially according to previously described procedures (Wu et al., 2009; Shang et al., 2010).

Ten-day-old Arabidopsis seedlings were harvested, ground in liquid nitrogen, then transferred into an Eppendorf tube containing ice-cold extraction buffer composed of 50 mM TRIS-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.1% Triton X-100, 10% glycerol, and 1× protease inhibitor cocktail (Roche). The sample was extracted for 15 min in ice, and centrifuged three times for 10 min each at 16,000 g; the supernatant was transferred to a new Eppendorf tube and centrifuged again at 12,000 g for 20 min, and then the concentration of the supernatant was detected by Coomassie Brilliant Blue G-250 (Amresco). The samples were either kept at 0 °C for immediate use or frozen and stored at −80 °C until use.

For the immunoblotting assays, proteins were separated by SDS-PAGE on 10% polyacrylamide gels, and transferred to nitrocellulose membranes (0.45 μm; Amersham Life Science) in a medium consisting of 25 mM TRIS-HCl (pH 8.3), 192 mM glycine, and 20% (v/v) methanol. After rinsing in TRIS-buffered saline (TBS) containing 10 mM TRIS-HCl (pH 7.5) and 150 mM NaCl, the blotted membranes were pre-incubated for 3 h in a blocking buffer containing 3% (w/v) bovine serum albumin dissolved in TBS supplemented by 0.05% (v/v) Tween-20 (TBST1) and then incubated with gentle shaking for 2 h at room temperature with appropriate antibodies. The anti-GFP serum (mouse, YTHX Biotechnology Beijing Limited Company, http://www.ythxbio.com/) and anti-actin serum (rabbit) were diluted 1:3000, and the anti-SOAR1 serum (rabbit) was diluted 1:2000 in the blocking buffer. Following extensive washes by TBST1, the membranes were incubated with goat anti-rabbit (or anti-mouse for GFP immunoblot) IgG (Cell Signaling Technology, Immunoblot) conjugated with alkaline phosphatase (diluted 1:10000 in TBST1) at room temperature for 1 h and then washed with TBST2 [50 mM TRIS-HCl, pH 7.5, 150 mM NaCl, and 0.1% (v/v) Tween-20] and TBS. The locations of antigenic proteins were visualized by incubating the membranes with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate.

Nucleus and mitochondrion staining in Arabidopsis roots

The roots of the 7-day-old OE1 seedlings were sampled and stained for 30 min in 1 μg ml⁻¹ 4',6-diamidino-2-phenylindole (DAPI; Sigma; a nuclear marker), or 15 min in 0.25 μM Mito Tracker Red CMXRos (Invitrogen). DAPI was dissolved in ddH₂O directly. The Mito Tracker Red CMXRos (a mitochondrion marker) stock solution (250 μM) was dissolved in dimethylsulphoxide, then diluted 1000 times with ddH₂O or 10 mM phosphate-buffered saline (PBS) (pH 7.4, 10 mM NaHPO₄, 10 mM NaH₂PO₄, 8.5 g l⁻¹ NaCl) when used for staining. After the staining process, the samples were rinsed several times with ddH₂O (for DAPI staining) or 10 mM PBS (for the Mito Tracker Red staining). Samples were examined with a Leica TCS SP5 confocal microscope under a 63.0 × 1.40 oil immersion objective.

Phenotypic analysis

Phenotypic analysis was carried out essentially as previously described (Shen et al., 2006; Wu et al., 2009; Shang et al., 2010). Seeds were harvested and stored at room temperature for 3–6 months before being used in the experiments. To assay germination and post-germination growth, the MS medium (Sigma-Aldrich, St Louis, MO, USA; full-strength MS) contained 3% (w/v) sucrose and 0.8% (w/v) agar, pH 5.8–6.0, and was supplemented or not with different concentrations of (±)-ABA. The seeds were sown and stratified in the MS medium at 4 °C for 3 d, and then they were placed at 20 °C under light conditions. Germination (emergence of radicals) was scored at the indicated times. Seedling growth was assessed by directly sowing the seeds in ABA-containing MS medium to investigate the response of seedling growth to ABA after germination. Another method was used to assay seedling growth in response to ABA: seeds were germinated after stratification on common MS medium and transferred to MS medium supplemented with different concentrations of (±)-ABA. The time for transfer was 48 h or 4 d (as indicated) after stratification. Seedling growth was investigated at the indicated times after the transfer.

Results

Down-regulation of SOAR1 increases, and up-regulation of SOAR1 abolishes, ABA sensitivity in seed germination and post-germination growth

The ABAR overexpression lines show ABA-hypersensitive phenotypes (Shen et al., 2006; Wu et al., 2009; see also the Materials and methods). From the ABAR overexpression lines (see the Materials and methods), which were identified by PCR analysis, a putative soar1 mutant named soar1-1D was screened, which showed an ABA-insensitive phenotype (Fig. 1A). PCR analysis showed that the construct for overexpressing the ABAR gene was inserted into the promoter of the SOAR1 gene in the soar1-1D mutant (Supplementary Fig. S1 at JXB online). The SOAR1 gene (At5g13110) encodes a PPR protein with tandem arrays of 10 predicted PPR motifs (Supplementary Fig. S2). The SOAR1 protein appears not to be a P-type member of the PPRs as it has a C-terminal extension (Supplementary Fig. S2) which is not related to other domains of PPRs according to its sequence. The GFP-tagged, functional, truncated ABAR was detected by immunoblot analysis in this soar1-1D mutant (Fig. 1B). To characterize the mutant further, the antibody against a truncated SOAR1 protein (303 amino acid residues from 299 to 602, see the Materials and methods) was generated, which was shown to be specific for SOAR1 (Supplementary Fig. S3). Immunoblot assays by using this anti-SOAR1 serum showed that the amount of SOAR1 protein in the soar1-1D mutant was enhanced >2-fold compared with the wild-type plants (Fig. 1B), which results from this ‘T-DNA’ construct for overexpressing the ABAR gene) insertion (Supplementary Fig. S1). The soar1-1D is a dominant allele, of which both the homozygous and heterozygous progeny showed strong ABA-insensitive phenotypes in ABA-induced seed germination inhibition and post-germination growth arrest (Fig. 1A–E). Further, T-DNA insertion mutant alleles, soar1-2 and soar1-3, which down-regulate the SOAR1 expression level were obtained (Fig. 1B; Supplementary Fig. S1). A null allele of the SOAR1 gene was not isolated, probably because the loss-of-function soar1 mutant is lethal. It was observed that, in contrast to the soar1-1D mutant, the soar1-2 and soar1-3 mutants showed ABA-hypersensitive phenotypes in ABA-induced seed germination inhibition and post-germination growth arrest (Fig. 1C–E; Supplementary Fig. S4A–E). The seeds of the soar1-2 and soar1-3 mutants germinated more slowly than the wild-type seeds in the exogenous ABA-free medium, suggesting that these mutant seeds may be overly sensitive to the endogenous...
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Fig. 1. SOAR1 negatively regulates ABA signalling. (A) Screen of the soar1-1D mutant. AO1–AO8 indicate the ABAR overexpression lines 1–8. The seeds were directly planted in 1 μM ABA-containing MS medium, and seedling growth was investigated 10 d after stratification. (B) Immunoblotting assays for the SOAR1–GFP fusion (95 kDa) and SOAR1 protein (68 kDa) levels in the 10-day-old seedlings of the soar1-1D, soar1-2, and soar1-3 mutants, and SOAR1 overexpression lines (OE1, OE3, and OE6). The SOAR1–GFP fusion and SOAR1 protein amounts were evaluated by scanning the protein bands, and relative band intensities, normalized relative to the band intensity (as 100%) from the sample of the OE1 (for the SOAR1–GFP fusion) or the wild-type Col plants (for the SOAR1 protein), are indicated by numbers above the bands. Actin was used as a loading control. In the soar1-1D mutant, the expression of the truncated ABAR (ABAR370C) tagged by GFP (67 kDa), introduced by transgenic manipulation, was tested, and the 150 kDa wild-type ABAR was also detected. (C) Seed germination: germination rates of the wild-type Col, soar1-1D, soar1-2, and soar1-3 mutants, and three SOAR1 overexpression lines (OE1, OE3, and OE6) were recorded on ABA-free (0 μM) and ABA-containing (0.6, 1, or 3 μM) MS medium from 24 h to 60 h after stratification. (D) Early seedling growth: seeds from the wild types Col and Ler, the abi1-1 dominant mutant, the abi1-3 abi2-2 double-knockout mutant, abi4-1 and abi5-1 mutants, and the different genotypes as described in (C) were directly planted in the MS medium supplemented with 0 (top), 0.5 (middle), or 10 μM (±)ABA (bottom), and the growth was investigated 10 d after stratification. (E) Statistical values of the early seedling growth described in (D) from the wild-type Col, soar1-1D, soar1-2, and soar1-3 mutants, and three SOAR1 overexpression lines (OE1, OE3, and OE6) in the
ABA at a low, physiological level (Fig. 1C; Supplementary Fig. S4A available at JXB online). The intensity of the ABA-hypersensitive phenotypes of the soar1-2 and soar1-3 mutants was similar to, or stronger than, that of the well-characterized abi1 abi2 double-knockout mutant.

SOAR1-overexpressing (OE) lines, in which SOAR1 was fused with GFP, were also generated. Immunoblot analysis detected both the natural SOAR1 and the SOAR1–GFP fusion protein in these OE lines (Fig. 1B; Supplementary Fig. S5B at JXB online). The OE lines showed strong ABA-insensitive phenotypes in ABA-induced seed germination inhibition and post-germination growth arrest (Figs 1C–E, 2A, B; Supplementary Figs S5A, S6A–D). The intensity of ABA-insensitive phenotypes of the OE lines was much stronger than that of the abi1-1 dominant mutant, abi4 and abi5 loss-of-function mutants, and a strong ABI2-overexpressing line ABI2-OE (Figs 1C–E and 2A, B; Supplementary Figs S5A, S6A–D), all of which are well-characterized strong ABA-insensitive mutants (Gosti et al., 1999; Leung et al., 1997; Finkelstein et al., 1998; Finkelstein and Lynch, 2000; Sun et al., 2011). It is noteworthy that the seeds of the SOAR1 OE lines germinated and their post-germination seedlings continued to grow in the medium containing >200 μM (±) ABA, while the wild-type Col seeds generally do not germinate in the 100 μM ABA medium. The bottom panel shows the pictures of early seedling growth of these genotypes. Each value in (C) and (E) 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 the germination rates among different genotypes at the same time point after stratification (C) or comparing the root lengths among different genotypes in the ABA-free and 0.6 μM ABA-containing medium (E).
SOAR1 overexpression suppresses ABA hypersensitive phenotypes of the ABAR overexpression lines

The SOAR1 and ABAR double overexpression lines were generated by using an ABAR overexpression line that expressed a truncated ABAR and showed ABA-hypersensitive phenotypes as described previously (Wu et al., 2009). Immunoblot analysis detected the truncated ABAR and SOAR1–GFP fusion proteins in the SOAR1 and ABAR double overexpression lines (Fig. 3A). These double overexpression lines showed strong ABA-insensitive phenotypes in ABA-induced seed germination inhibition and post-germination growth arrest, which were similar to the SOAR1 overexpression lines (Fig. 3B–E). These findings, together with the discovery of the soar1-1D dominant mutant (Fig. 1A, B), reveal that SOAR1 functions downstream of ABAR in the ABA signalling pathway.

The SOAR1 gene is expressed in the whole plant but preferentially in seeds, and the SOAR1 protein is localized to both the cytosol and nucleus

The gene expression data in the public websites at http://bar.utoronto.ca and http://www.genevestigator.com showed that the SOAR1 gene is expressed in different organs/tissues, with the highest level in seeds, and this expression profile was confirmed with the SOAR1 promoter–GUS transgenic lines (Supplementary Fig. S9 at JXB online).

As regards the subcellular localization of SOAR1, a bioinformatics search allowed the prediction that SOAR1 may localize to the mitochondrion, chloroplast, or nucleus (Supplementary Fig. S10 at JXB online). The transient expression assays in Arabidopsis protoplasts showed that SOAR1 co-localized with the cytosol–nucleus dual-localized PYR1 (Fig. 4A), which is a member of the PYR/PYL/RCAR receptors for ABA (Ma et al., 2009; Park et al., 2009; Santiago et al., 2009), while the SOAR1 fluorescence was not seen in the chloroplasts (Fig. 4A). However, the cytosolic SOAR1 disappeared, and the SOAR1 fluorescence was seen only in the nucleus and co-localized with a nuclear marker, bHLH (basic helix–loop–helix) transcription factor FBI1 (At1g02340; Fairchild et al., 2000; Jang et al., 2005) when an N-terminal 35 amino acid fragment was deleted (Fig. 4A), suggesting that the N-terminal 35 amino acid fragment is required for the cytosolic localization of the SOAR1 protein. The data from the transient expression assays in onion epidermis cells are consistent with those from the transgenic Arabidopsis protoplasts (Fig. 4B).

The localization of SOAR1 was further verified by immunoblot assays with the purified cytosolic and nuclear fractions from Arabidopsis, in which the cytosolic marker could not be detected in the nuclear fraction and the nuclear marker histone H3 could not be detected in the cytosolic fraction (Fig. 4C, D), showing the purity of the cytosolic and nuclear fraction. Using the antibody specific for SOAR1 (Supplementary Fig. S3 at JXB online), we immunodetected the SOAR1 protein in both the purified cytosolic and nuclear fractions (Fig. 4C, D), which consistently confirmed that SOAR1 is a cytosol and nucleus dual-localized protein.

It is noteworthy that, in the proteins sampled from the germinating seeds 24 h after stratification, a form of SOAR1 protein was detected in the nuclear fraction with a higher molecular mass (74 kDa) than the normal protein in the cytosolic fraction (68 kDa) (Fig. 4C); and in the proteins sampled from the 2-week-old seedlings, both forms of SOAR1 protein were detected in the nuclear fraction with molecular masses of 68 kDa and 74 kDa, respectively (Fig. 4D). However, only a very weak signal of the 74 kDa protein was detected that often could be scarcely seen in the total proteins (Fig. 4C, D) probably because of too low a concentration of the SOAR1 in the total protein extracts. These data indicate that the SOAR1 protein is subjected to a post-translational modification before or after it enters the nucleus, which may be associated with its function in the nucleus. This aspect remains an open question and needs further studies in the future.

Given that SOAR1 protein is present in the cytosolic space surrounding the mitochondria, it was further tested whether SOAR1 resides in the mitochondrion by using the above-mentioned SOAR1–GFP transgenic line OE1 (Fig. 1A). The nuclear localization of the SOAR1–GFP fusion protein was first verified in the root of the OE1 plants, as visualized by co-localization of the GFP fluorescence with the DAPI-stained nuclei (Fig. 4E). It was further shown that the localization pattern of the SOAR1 protein visualized by GFP fluorescence is distinct from the distribution profile of the mitochondria labelled by a mitochondrial marker (MitoTracker Red) (Fig. 4F). Taken together, these data demonstrate that SOAR1 localizes to the cytosol and nucleus, but not to chloroplasts or mitochondria.

Changes in SOAR1 expression after expression of a subset of ABA-responsive genes

The expression levels of a subset of the ABA-responsive genes were tested in the soar1-2 mutant and the SOAR1
Fig. 3. SOAR1 acts genetically downstream of ABAR and upstream of ABI5 in ABA signalling. (A) Immunoblot analysis of the SOAR1–GFP fusion, SOAR1, ABAR, and truncated ABAR (ABAR\textsubscript{370C}) proteins in 10-day-old seedlings of the wild-type Col, ABAR overexpressor (ABARo), SOAR1 overexpressor (OE1), and ABARo/OE1 double overexpression line. Actin was used as a loading control. (B) Seed germination rates of the different genotypes described in (A), which were recorded on ABA-free (0 μM) and ABA-containing (1 μM) MS medium from 24 h to 60 h after stratification. (C–E) Early seedling growth of the different genotypes described in (A). Seeds were directly planted in MS medium supplemented with 0 or 1 μM (±) ABA; the growth was investigated (C and D) and root length (E) was measured 10 d after stratification. (F) Immunoblot analysis of the SOAR1 protein in 10-day-old seedlings of the wild-type Col, abi5-1, soar1-2, and the soar1-2 abi5-1 double mutant. Actin was used as a loading control. (G) Seed germination rates of the different genotypes described in (F), which were recorded on ABA-free (0 μM) and ABA-containing (1 μM) MS medium from 24 h to 60 h after stratification. (H–J) Early seedling growth of the different genotypes described in (F). Seeds were directly planted in MS medium supplemented with 0 or 0.6 μM (±) ABA; the growth was investigated (H and I) and root length (J) was measured 10 d after stratification. Each value in (B), (E), (G), and (J) 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 the germination rates among different genotypes at the same time point after stratification (B, G) or comparing the root lengths among different genotypes in the ABA-free and ABA-containing medium (E, J).
SOAR1 regulates ABA signalling overexpressor OE1. These genes include ABF4/AREB2 (Choi et al., 2000; Uno et al., 2000), ABI1 (Leung et al., 1994; Meyer et al., 1994; Gosti et al., 1999), ABI2 (Leung et al., 1997), ABI3 (Giraudat et al., 1992), ABI4 (Finkelstein et al., 1998), ABI5 (Finkelstein and Lynch, 2000), DREB1A, DREB2A (Liu et al., 1998), MYB2 (Abe et al., 2003), PYRI/
RCAR11, PYL2/RCAR14, PYL4/RCAR10, PYL7/RCAR2, PYL9/RCAR1 (Ma et al., 2009; Park et al., 2009), RD29A, RD29B (Yamaguchi-Shinozaki and Shinozaki, 1994), RAB18 (Lang and Palva, 1992), SnRK2.2, and SnRK2.3 (Fujii et al., 2007; Fujii and Zhu, 2009). The expression of the positive ABA signalling regulator-encoding genes (or positively ABA-responsive genes) ABI3, ABI4, ABI5, ABF4, DREB2A, PYR1, RAB18, RD29A, RD29B, SnRK2.2, and SnRK2.3 was significantly up-regulated in the soar1-2 mutant, while it was repressed in the OE1 line (Fig. 5A–D). However, the expression levels of the other positive ABA signalling regulator-encoding genes PYL2, PYL4, DREB1A, and MYB2 was not changed much in the soar1-2 mutant and the OE1 line compared with the wild-type plants, though some significant differences were still detected (Fig. 5B). The expression of another two genes encoding ABA receptors was altered differently: PYL9 was remarkably repressed, while PYL7 was significantly up-regulated in the soar1-2 mutant (Fig. 5B). It is noteworthy that the ABI3 and ABI5 genes, encoding two critical, positive regulators of ABA-responsive seed germination and post-germination growth, were substantially suppressed to a null level in the OE1 line while they were considerably up-regulated in the soar1-2 mutant (Fig. 5A, D), suggesting that these genes are potential, main targets of the SOAR1 protein. The expression of the negative ABA signalling regulator-encoding gene ABI1 was significantly repressed in the soar1-2 mutant, while ABI2 was remarkably up-regulated in the OE1 line (Fig. 5A, C).

The exogenous ABA treatment enhanced the expression levels of the ABA-responsive genes including ABI1, ABI2, ABI3,
Loss of function of ABI5 suppresses ABA-hypersensitive phenotypes of the soar1-2 T-DNA insertion mutant

Given that the ABI5 transcription factor is a key player regulating seed germination and post-germination growth in response to ABA (Finkelstein and Lynch, 2000; Lopez-Molina et al., 2001), and the ABI5 gene expression is significantly up-regulated in the soar1-2 mutant and nearly knocked out in the OE1 line (Fig. 5C), it was tested whether ABI5 functions downstream of SOAR1 as a potential target of this PPR protein. The abis5-1 soar1-2 double mutant was generated, in which abis5-1 is a knockout allele of the ABI5 gene and soar1-2 a knockdown allele of the SOAR1 gene. The abis5-1 soar1-2 double mutant showed ABA-insensitive phenotypes in ABA-induced seed germination inhibition and post-germination growth arrest, which are similar to the abis5-1 mutant (Fig. 3F–J). These findings suggest that SOAR1 may function upstream of ABI5 in the ABA signalling pathway.

Discussion

SOAR1 is a critical, negative, regulator acting downstream of ABAR and probably upstream of ABI5 in ABA signalling

It was shown that down-regulation of SOAR1 strongly increases, and up-regulation of SOAR1 almost completely impairs, ABA sensitivity in seed germination and post-germination growth. The intensity of the ABA overly-sensitive phenotypes of the two mutants was similar to, or stronger than, that of the well-characterized abil ab2 double-knockout mutant (Fig. 1; Supplementary Fig. S4 at JXB online). It could be expected that a complete loss of SOAR1 function would lead to even stronger ABA-hypersensitive phenotypes or dormant seeds; however, the null allele of the SOAR1 gene is likely to be lethal. The intensity of ABA-insensitive phenotypes of the SOAR1-overexpressing lines was much stronger than that of the abil-1 dominant mutant, ab4 and ab5 loss-of-function mutants, and a strong AB2-overexpressing line ABI2-OE (Figs 1, 2; Supplementary Figs S5, S6). In particular, it is surprising to note that the seeds of the SOAR1 overexpressors germinated and their post-germination seedlings continued to grow in the medium containing >200 μM (±) ABA (Fig. 2; Supplementary Figs S5, S6), and the 48-hour-old germinating seeds of the SOAR1 overexpressors even grew in the medium containing >500 μM (±)ABA (Fig. 2). Previous studies reported that the seeds of the srk2dei mutant, a triple-knockout mutant of three SnRK2 members SnRK2.2, SnRK2.3, and SnRK2.6, germinated and continued to grow in the presence of 50 μM or 100 μM exogenous ABA, which is believed to impair the ABA response completely (Fujii and Zhu, 2009; Nakashima et al., 2009; Umezawa et al., 2009). In this regard, the intensity of ABA insensitivity of the SOAR1 overexpression lines in this study is comparable with that of the triple loss-of-function mutant of the SnRK2 members (Fig. 2; Supplementary Figs S5, S6). However, the seeds of the SOAR1 overexpression lines are not viviparous despite their strong ABA insensitivity, suggesting that there may be a sophisticated mechanism to balance and optimize the function of SOAR1 under basal growth conditions.

All the findings reveal that SOAR1 is a critical, negative, regulator of ABA signalling, which regulates key processes of cell signalling in response to ABA. The ABA hypersensitivity of the ABAR overexpressors was suppressed by up-regulation of SOAR1 (Figs 1, 5), showing that SOAR1 functions in ABA signalling downstream of ABAR. Loss of function of ABI5 suppressed ABA-hypersensitive phenotypes of a SOAR1 knockdown mutant (soar1-2), suggesting that SOAR1 may function upstream of ABI5. These findings suggest a possible ABAR–SOAR1–ABI5-linked signalling cascade in the ABA signalling pathway, though it still remains unknown whether a direct interconnection exists between ABAR and SOAR1 or between SOAR1 and ABI5.

How does SOAR1 work in ABA signalling?

Currently, it remains largely unknown whether and how the PPR proteins regulate nuclear gene expression. In the two identified Arabidopsis nucleus-localized PPR proteins, GRP23 interacts physically with RNA polymerase II, suggesting its potential function as a transcription regulator (Ding et al., 2006); PNM1, dual localized to both the nucleus and mitochondrion, physically interacts with the nucleosome assembly protein NAP1 and the transcription factor TCP8, suggesting its potential roles in the coordination of

\[ \text{ABF4, DREB1A, DREB2A, MYB2, PYL7, RAB18, RD29A, RD29B, and SnRK2.3 in the wild-type Col plants, whereas such ABA responsiveness of gene expression was significantly altered in the soar1-2 mutant and OE1 line (Fig. 5A–C). It is noteworthy that, with the ABAR treatment, the expression levels of ABI3 and ABI5 still remained lower in the OE1 line (Fig. 5A, D), and the levels of most of the positive ABA signalling regulator-encoding genes such as ABI3, ABF4, ABI5, ABF4, DREB2A, PYL7, SnRK2.2, and SnRK2.3 remained higher in the soar1-2 mutant than in wild-type plants (Fig. 5A–D). The low expression of ABI1 was not significantly changed by ABA treatment in the soar1-2 mutant, and an even higher level of ABI2 was observed in the ABA-treated OE1 line (Fig. 5A, C).} \]
mitochondrial and nuclear gene expression (Hammani et al., 2011). However, whether and how these nuclear PPR proteins regulate nuclear gene expression, and what their downstream, nuclear target genes are, have not been reported (Ding et al., 2006; Hammani et al., 2011).

In the present experiments, it was observed that down-regulation of the SOAR1 gene enhanced, but up-regulation of the SOAR1 gene repressed, the expression levels of the ABA-responsive genes ABI3, ABI4, ABI5, ABI4, DREB2A, PYR1, RAB18, RD29A, RD29B, SnRK2.2, and SnRK2.3. It is particularly noteworthy that the expression levels of the ABI3, ABI5, RAB18, and RD29B genes were markedly increased, while the level of ABI1 was almost completely suppressed by down-regulation of the SOAR1 gene, and the ABI3 and ABI5 genes were almost completely suppressed by overexpression of the SOAR1 gene (Fig. 5). It is also surprising to observe that the level of ABI2 expression was increased by >100-fold with up-regulation of the SOAR1 gene (Fig. 5). The marked increase of the ABI2 mRNA in the SOAR1 overexpression lines is likely to be caused by a decrease of some repressive factors of ABI2 to which is SOAR1 targeted, protecting the ABI2 mRNA from degradation. An ABA receptor member-encoding gene PYL9 was repressed, while the gene of another member PYL7 was up-regulated in the soar1-2 mutant (Fig. 5), suggesting a SOAR1-related balance mechanism that may function in RNA processing to maintain homeostasis of these family proteins. These gene expression data strongly suggest that these genes are most probably potential, direct or indirect targets of the SOAR1 protein, and explain, at least partly, the strong ABA-related phenotypes of the soar1 mutants and SOAR1 overexpressors. The gene expression profile under treatment with exogenous ABA is consistent with this conclusion. Genetic evidence that ABI5 may function downstream of SOAR1 (Fig. 3) strongly supports that ABI5 mRNA may be a target of the SOAR1 protein, which may function as a SOAR1–ABI5 directly coupled signalling module in the ABA signalling pathway.

It remains largely unclear whether SOAR1 participates in the PYR/PYL/RCAR-mediated ABA signalling pathway, a well-characterized core ABA signalling pathway (Fuji et al., 2009; Ma et al., 2009; Park et al., 2009; Santiago et al., 2009; Cutler et al., 2010). However, the expression data of the ABA-responsive genes in the soar1-2 mutant and SOAR1 overexpressor OE1 (Fig. 5) showed that the alteration in SOAR1 expression significantly changes the expression of a subset of genes, of which the encoded proteins have been identified to be directly involved in the PYR/PYL/RCAR-mediated ABA signalling, including the PYR/PYL/RCAR family members PYR1/RCAR11, PYL7/RCAR2, and PYL9/RCAR1, and some key signalling components ABI1, ABI2, SnRK2.2, SnRK2.3, ABI5, and ABI4 (Fig. 5). If the ABI5 mRNA is a direct target of SOAR1, ABI5 may be a common target of SOAR1 and SnRK2.2/3/6 in the ABA signalling pathway where the SnRK2 members regulate ABI5 by a phosphorylation process, a post-translation modification (Fujii et al., 2007; Fuji and Zhu, 2009), while SOAR1 may regulate ABI5 by post-transcriptional RNA processing. Thus, they may cooperate to regulate ABA signalling. Additionally, the gene expression data suggest that SOAR1 may regulate RNA processing of other key signalling component-encoding genes including SnRK2.2 and SnRK2.3. All the gene expression data support the idea that SOAR1 may also be involved in the PYR/PYL/RCAR-mediated ABA signalling which may possibly interact with ABAR/CHLH-mediated signalling through SOAR1. However, whether and how PYR/PYL/RCAR function as ABA receptors to regulate SOAR1, and how PYR/PYL/RCAR-mediated signalling interacts with ABAR/CHLH-mediated signalling through SOAR1, need further studies. Exploration of the detailed mechanisms of the cytosol-nuclear dual-localized SOAR1 protein that functions in the nuclear events as a critical component of ABA signalling, such as the nuclear mechanism by which SOAR1 regulates ABI5 mRNA processing, will be of particular importance in the future to understand the functional mechanism of PPR proteins and the highly complicated ABA signalling network.

Supplementary data
Supplementary data are available at JXB online.

Figure S1. Diagrams of the three T-DNA insertion mutants in the SOAR1 gene (At5g11310).

Figure S2. SOAR1 is a member of the pentatricopeptide repeat protein family.

Figure S3. Test of the specificity of the anti-SOAR1 serum.

Figure S4. The soar1-2 and soar1-3 mutants are hypersensitive to ABA in seed germination and early seedling growth.

Figure S5. The ABA-insensitive phenotypes in early seedling growth of 12 SOAR1 overexpression lines.

Figure S6. Phenotypes of the soar1-1D, soar1-2, and soar1-3 mutants, and SOAR1 and ABI overexpression lines in response to ABA.

Figure S7. Transgenic expression of SOAR1 rescues the ABA-hypersensitive phenotypes of the soar1-2 and soar1-3 mutant.

Figure S8. ABA concentrations in the germinating seeds of different genotypes.

Figure S9. SOAR1 is expressed in different organs/tissues, with the highest expression level in seeds.

Figure S10. Prediction of the subcellular localization of SOAR1 protein.

Table S1. Primers used in this study.

Acknowledgements
We thank Drs Yan Guo and Zhong-Jun Zhang (China Agricultural University, Beijing, China), and Drs Dong Liu and Li Yu (Tsinghua University, Beijing, China) for help with materials and equipment. This research was supported by the National Key Basic Research Program of China (2012CB114300-002), the National Natural Science Foundation of China (grant nos 31200213 and 31170268), and the Ministry of Agriculture of China (grant no. 2013ZX08009003).

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