RESEARCH PAPER

A link between magnesium-chelatase H subunit and sucrose nonfermenting 1 (SNF1)-related protein kinase SnRK2.6/OST1 in Arabidopsis guard cell signalling in response to abscisic acid

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Abstract

Magnesium-chelatase H subunit [CHLH/putative abscisic acid (ABA) receptor ABAR] positively regulates guard cell signalling in response to ABA, but the molecular mechanism remains largely unknown. A member of the sucrose non-fermenting 1 (SNF1)-related protein kinase 2 family, SnRK2.6/open stomata 1 (OST1)/SRK2E, which plays a critical role in ABA signalling in Arabidopsis guard cells, interacts with ABAR/CHLH. Neither mutation nor over-expression of the ABAR gene affects significantly ABA-insensitive phenotypes of stomatal movement in the OST1 knockout mutant allele srk2e. However, OST1 over-expression suppresses ABA-insensitive phenotypes of the ABAR mutant allele cch in stomatal movement. These genetic data support that OST1 functions downstream of ABAR in ABA signalling in guard cells. Consistent with this, ABAR protein is phosphorylated, but independently of the OST1 protein kinase. Two ABAR mutant alleles, cch and rtl1, show ABA insensitivity in ABA-induced reactive oxygen species and nitric oxide production, as well as in ABA-activated phosphorylation of a K+ inward channel KAT1 in guard cells, which is consistent with that observed in the pyr1 pyl1 pyl2 pyl4 quadruple mutant of the well-characterized ABA receptor PYR/PYL/RCAR family acting upstream of OST1. These findings suggest that ABAR shares, at least in part, downstream signalling components with PYR/PYL/RCAR receptors for ABA in guard cells; though cch and rtl1 show strong ABA-insensitive phenotypes in both ABA-induced stomatal closure and inhibition of stomatal opening, while the pyr1 pyl1 pyl2 pyl4 quadruple mutant shows strong ABA insensitivity only in ABA-induced stomatal closure. These data establish a link between ABAR/CHLH and SnRK2.6/OST1 in guard cell signalling in response to ABA.

Key-words: Abscisic acid signalling; Arabidopsis thaliana; guard cell; Mg-chelatase H subunit; SNF1-related protein kinase SnRK2.6/OST1; stomatal movement.

Introduction

In higher plants, stomatal pores formed by a pair of guard cells play key roles in allowing photosynthesis and transpiration. Through controlling stomatal opening and closure, the plants regulate gas exchange and water loss, which is directly related to the turgor of guard cells. The change of turgor is modulated by the dynamic changes in intracellular concentration
of ions and sugars (Archana et al., 2011). Different channels and transporters are involved in ion flux across membranes mediated by phytohormone abscisic acid (ABA) signalling. In response to water deficit, ABA is synthesized and released from storage, and then serves as an endogenous messenger to promote stomatal closure.

In recent years, significant progress has been made in understanding ABA signalling of guard cells. Many signalling components have been identified, including a central regulator open stomata 1 (OST1, also known as SnRK2.6 or SRK2E), a member of the sucrose nonfermenting 1 (SNF1)-related protein kinase 2s family (Mustilli et al., 2002; Yoshida et al., 2002). Different from its homologues SnRK2.2 and SnRK2.3, which regulate mainly seed germination and seedling growth by activating ABA-responsive bZIP transcription factor AFB (Boudsocq et al., 2004; Kobayashi et al., 2004; Furihata et al., 2006; Yoshida et al., 2006; Fujii et al., 2007; Fujii and Zhu, 2009; Fujii et al., 2009), OST1 is preferentially expressed in guard cells, and the OST1 gene mutant shows impaired ABA-induced stomatal closure, revealing that OST1 acts as a positive regulator of guard cell signalling in response to ABA (Mustilli et al., 2002; Yoshida et al., 2002).

OST1 phosphorylates the inward K⁺ channel KAT1, and the C-terminal region of KAT1 is the direct phosphorylation target domain of OST1 (Sato et al., 2009; Acharya et al., 2013). Phosphorylation of KAT1 leads to inhibition of its activity to drive inward K⁺ flux, which is required for ABA-induced stomatal closure and inhibition of stomatal opening (Kwak et al., 2001; Pandey et al., 2007). ABA inhibition of inward K⁺ channels and light-induced stomatal opening are reduced in ost1 mutants, while transgenic plants overexpressing OST1 show ABA hypersensitivity in these responses, suggesting that OST1 negatively regulates KAT1 to induce stomatal closure and inhibit stomatal opening in response to ABA (Acharya et al., 2013). These observations reveal that KAT1 is a node of the OST1-mediated ABA signalling cascades in guard cells.

Slow (S-type) anion channel associated 1 (SLAC1) is another member of the OST1-mediated ABA signalling cascades in guard cells. Slow (S-type) anion channel associated 1 (SLAC1) is another member of the OST1-mediated ABA signalling cascades in guard cells. Phosphorylation of KAT1 leads to inhibition of its activity to drive inward K⁺ flux, which is required for ABA-induced stomatal closure and inhibition of stomatal opening (Kwak et al., 2001; Pandey et al., 2007; Geiger et al., 2009, 2010; Lee et al., 2009, 2013; Brandt et al., 2012; Acharya et al., 2013). Genetic evidence supports that SLAC1, together with KAT1, plays critical roles in OST1-mediated guard cell signalling in response to ABA (Geiger et al., 2009; Acharya et al., 2013). In addition, OST1 phosphorylates a K⁺ uptake transporter KUP6 (Osakabe et al., 2013), and regulates ABA activation of rapidly activating (QUAC1) anion currents in guard cells (Imes et al., 2013), which may also be involved in the mechanism of OST1-mediated ABA signalling in guard cells.

ABA accumulation in guard cells triggers the generation of reactive oxygen species (ROS) (Pei et al., 2000; Zhang et al., 2001). ROS production is located downstream of OST1 in the ABA signalling of guard cells (Mustilli et al., 2002; Acharya et al., 2013), where ABA-activated OST1 interacts with and phosphorylates two NADPH oxidases, AtrbohD and AtrbohF, which play key roles in ABA-induced ROS generation in Arabidopsis guard cells (Kwak et al., 2003; Acharya et al., 2013). ROS serves as a second-messenger molecule regulating stomatal channels and transporters to mediate ABA signalling in guard cells. Exogenous ROS suppresses the inward K⁺ channel in Vicia guard cells (Zhang et al., 2001). ROS also stimulates Ca²⁺ release from internal stores and influx across the plasma membrane, and then promotes stomatal closure (Pei et al., 2000). Another second-messenger molecule—nitric oxide (NO)—also plays a positive role in ABA-induced stomatal closure (Neill et al., 2002). The level of NO in guard cells increases dependently on the quick burst of ROS (Bright et al., 2006), and NO may possibly function by targeting inward K⁺ and anion channels in the same way as ROS (Garcia-Mata et al., 2003). NO also modulates guard cell signalling through the generation of nitrated cGMP (Joudoi et al., 2013). A recent study reported that ABA-induced NO causes S-nitrosylation of OST1 and blocks its kinase activity, thereby regulating the ABA signalling pathway via negative feedback (Wang et al., 2015).

Recent progress has established an ABA signalling pathway in guard cells from primary events to activation of different channels. Clade A protein phosphatase 2Cs (PP2Cs) bind to, dephosphorylate, and inhibit kinase activity of OST1, negatively regulating ABA signalling (Mustilli et al., 2002; Yoshida et al., 2006; Fujii et al., 2009; Umezawa et al., 2009; Vlad et al., 2009, 2010; Cutler et al., 2010). The START-domain family proteins PYR/PYL/RCARs—the best characterized cytosolic ABA receptors (Ma et al., 2009; Park et al., 2009; Santiago et al., 2009; Cutler et al., 2010; Nishimura et al., 2010)—perceive the ABA signal, and bind to and inactivate PP2Cs, leading to release of OST1 from inhibition by PP2Cs (Mustilli et al., 2002; Yoshida et al., 2006; Fujii et al., 2009; Umezawa et al., 2009; Vlad et al., 2009, 2010; Cutler et al., 2010), activating downstream signalling components to finally relay the ABA signal to different ion channels, which modulates ion flux across guard cell membranes to induce stomatal closure (Kwak et al., 2001; Pandey et al., 2007; Geiger et al., 2009, 2010; Lee et al., 2009, 2013; Brandt et al., 2012; Acharya et al., 2013; Imes et al., 2013; Osakabe et al., 2013).

The magnesium-protoporphyrin IX chelatase large subunit (Mg-chelatase H subunit CHLH/putative ABA receptor ABAR) was reported to function as a candidate ABA receptor in the Arabidopsis chloroplast space, and an ABAR-mediated signalling pathway involving the chloroplast protein cochaperonin CPN20 and cytosolic-nuclear WRKY18/40/60 transcription repressors has been described (Shen et al., 2006; Wu et al., 2009; Shang et al., 2010; Du et al., 2012; Liu et al., 2012; Liu et al., 2013; Yan et al., 2013; Zhang et al., 2013, 2014; Wang and Zhang, 2014). Although whether it binds ABA remains controversial (Shen et al., 2006; Müller and Hansson, 2009; Wu et al., 2009; Tsuchuki et al., 2011; Wang et al., 2011; Du et al., 2012), increasing evidence supports that CHLH/ABAR plays a crucial role in ABA signalling for major ABA responses including ABA-inhibited seed germination and post-germination growth, as well as ABA-induced stomatal closure and inhibition of light-induced stomatal opening. Four abar mutant alleles in Arabidopsis, abar-2, abar-3, cch, and rtiI, display impaired ABA responses (Shen et al., 2006; Wu et al., 2009; Tsuchuki et al., 2011, 2013; Du et al., 2012). A recent genetic screen has
identified a pentatricopeptide repeat protein SOAR1 functioning as a hub of ABA signalling downstream of ABAR, and it was reported that SOAR1 over-expression almost completely impairs ABA responses, suggesting that ABAR mediates a central ABA signalling pathway (Jiang et al., 2014; Mei et al., 2014; Wang and Zhang, 2014).

In different systems including Arabidopsis, Nicotiana benthamiana, and Prunus persica leaves, ABAR was shown to positively mediate guard cell signalling in response to ABA (Shen et al., 2006; Legnaioli et al., 2009; Wu et al., 2009; Jia et al., 2011; Tsuzuki et al., 2011, 2013; Du et al., 2012; Zhang et al., 2013). Tsuzuki and co-workers (2013) recently showed that CHLH/ABAR mediates ABA inhibition of blue light (BL)-induced phosphorylation of H+-ATPase in Arabidopsis guard cells, suggesting that ABAR regulates not only ABA-induced stomatal closure but also ABA inhibition of BL-mediated stomatal opening. However, the molecular mechanism by which ABAR regulates stomatal movement in response to ABA remains largely unknown. In this study, SnRK2.6/OST1 is shown to be an interaction partner of ABAR, and functions downstream of ABAR in ABA signalling in Arabidopsis guard cells. Consistent with this point of view, ABAR was shown to share downstream signalling steps with the PYR/PYL/RCAR receptor for ABA in guard cells. These data establish a functional link between ABAR/CHLH and OST1 in guard cell signalling in response to ABA.

Materials and methods

Plant materials and growth conditions

Arabidopsis thaliana ecotype Columbia-0 (Col-0) was used to generate transgenic plants and as the wild-type control. To generate the SnRK2.6/OST1 (At4g33950) over-expression lines, the full-length sequence of OST1, amplified by PCR with the primers listed in Supplementary Table S1 (available at JXB online), was cloned into the binary vector pCAMBIA-1300-221, which was driven by the cauliflower mosaic virus (CaMV) 35S promoter. The construct was introduced into Arabidopsis ecotype Col-0 plants to generate the OST1 over-expression lines (OST1OE). The OST1 levels were analysed by quantitative real-time PCR. ABAR-over-expression lines were generated by introducing an ABAR gene (At5g13630) fragment [encoding a truncated ABAR with amino acids (aa) 631–1381, named ABAR631–1381] into Arabidopsis ecotype Col-0 plants, where ABAR631–1381 was fused with GFP protein, and the construct was driven by 35S promoter (Wu et al., 2009). It was previously shown that this C-terminal half of ABAR tagged with GFP functions similarly to full-length ABAR in transgenic plants, leading to ABA hypersensitivity in the major ABA responses; the intensities of ABA-hypersensitive phenotypes of the C-terminal half of ABAR-expressing lines are similar to those of full-length ABAR-transgenic plants (Wu et al., 2009). Therefore, the transgenic lines expressing this C-terminal half of ABAR were used to overexpress ABAR in this experiment. The cDNA isolation and transgenic manipulation were performed as previously described (Wu et al., 2009).

The cch mutant and the rtl1 mutant, two mutant alleles of the ABAR gene, were gifts from Dr J. Chory (The Salk Institute, La Jolla, CA, USA) and Dr T. Kinoshita (Nagoya University, Japan), respectively. The pyr1 pyl1 pyl2 pyl4 quadruple ABA receptor knockout mutant (Park et al., 2009) was a gift from Dr Cutler (University of California at Riverside, Riverside, CA, USA). The OST1 T-DNA insertion knockout mutant (SALK_008068) was obtained from Arabidopsis Biological Resource Center (ABRC), which was identified and described previously with the name srk2e (Yoshida et al., 2002; Umezawa et al., 2009). The cch srk2e double mutant, ABAR631–1381-over-expression lines under srk2e background (ABAR631–1381OE/srk2e), and OST1-over-expression lines under cch background (OST1OE-1/cch) were constructed by crossing. All the mutant lines were identified by PCR genotyping using the primers presented in Supplementary Table S1.

Arabidopsis seeds were disinfected and plated on MS medium (Sigma-Aldrich, St Louis, MO, USA) supplemented with 3% sucrose and 0.8% agar (pH 5.9), chilled for 3 d at 4 °C and transferred into a growth chamber at light intensity 80 μmol photons m−2 s−1 or into compost at 120 μmol photons m−2 s−1 using cool white fluorescent lamps under a 16 h light/8 h dark photoperiod and 60% relative humidity.

Yeast two-hybrid assays

Interaction between ABAR and OST1 was assayed by a yeast two-hybrid system as described by the manufacturer (Clontech, Mountain View, CA, USA). Three truncated ABAR proteins—the C-terminal half of ABAR (aa 692–1381, ABARc690), the N-terminal half of ABAR (aa 1–691, ABAR669), and the middle section of ABAR (aa 692–941, ABAR290)—were fused to GAL4 binding domain (BD) in the pGBK7T vector. The full-length sequence of OST1 was cloned into the prey plasmid pGADT7 with the DNA activation domain (AD). The primers used for constructing the related plasmids are listed in Supplementary Table S1. The different combinations of constructs were co-transformed to yeast strain AH109 and tested on SD screening medium as indicated for 5–7 d at 30 °C. To test α-galactosidase (α-Gal) activity of transformed yeast cells, substrate p-nitrophenyl-α-D-galactoside was dropped onto the transformed yeast cells to detect the colour changes according to the manufacturer’s protocols. The yeast co-transformed with the construct pair ABD (srb2e/ABAR669) and BD plus AD-OST1 were taken as negative controls. The yeast co-transformed with the construct pair BD-53 plus AD-T was taken as a positive control. For the CoIP assays in yeast, yeast strains were cultured on SD− medium to OD600 1.0 at 30 °C, and then cells were harvested and lysed with an extraction buffer containing 50 mM HEPES, pH 7.4, 10 mM EDTA, 0.1% (v/v) TritonX-100, 1 mM phenylmethylsulphonyl fluoride (PMSF), and 5 μg/mI protein inhibitor cocktail (Roche, Mannheim, Germany). Total protein was pre-cleared with the protein A/G plus beads (Santa Cruz Biotechnology, Dallas, TX, USA) and was divided into two parts. One was incubated with mouse anti-HA-tag antibody (MBL, Nagoya, Japan) and the other was incubated with pre-immune serum (MBL, Nagoya, Japan) for 1 h. After incubation, the protein A/G plus beads were added into the buffer and incubation continued at 4 °C for another 4 h. The beads were washed twice with buffer A containing 50 mM Tris (pH 8.0), 150 mM NaCl, and 0.1% (v/v) Triton X-100 and twice with buffer B containing 50 mM Tris (pH 8.0) and 0.1% (v/v) Triton X-100, and then resuspended in protein loading buffer. The immunoprecipitates were separated on a 10% SDS-PAGE and analysed by immunoblotting with anti-Myc serum.

 Luciferase complementation imaging assay

The luciferase complementation imaging (LCI) assay was used to further confirm ABAR and OST1 interaction in N. benthamiana leaves according to previously described procedures (Chen et al., 2008). The firefly luciferase (Luc) enzyme was divided into the N-terminal part (NLuc) and C-terminal part (CLuc). ABAR and OST1 were cloned into pCAMBIA-NLuc vector fused with NLuc and pCAMBIA-CLuc vector fused with CLuc, respectively. The primers used for constructing the related plasmids are listed in Supplementary Table S1. The constructs were transformed into A. tumefaciens strain GV3101. Using the A. tumefaciens-mediated transformation with equal concentrations and volumes, different
combinations of constructs were introduced to the fully expanded leaves of the 7-week-old *N. benthamiana* plants by a needleless syringe. The amounts of the constructs were kept the same among treatments and controls for each group of assays. After infiltration, plants were placed with 16 h light/8 h dark for 48 h at 24 °C. The Luciferase activity was observed by a cooled CCD imaging apparatus (Andor iXon, Andor Technology, Belfast, UK).

**Preparation of recombinant proteins in Escherichia coli**

To prepare recombinant OST1 and truncated KAT1 protein, the full-length ORF of OST1 and a KAT1 fragment encoding the truncated KAT1 (corresponding to the C-terminal region covering aa 301–677) were isolated using the primers listed in Supplementary Table S1, and cloned into pET-48b (+) vector (Novagen, Madison, WI, USA). The recombinant plasmids were expressed in *E. coli* strain BL21(DE3) as His-tagged fusion proteins. The *E. coli* strains were grown at 37 °C in LB medium until the OD 

**Stomatal movement assay**

The stomatal movement assay was performed essentially as described previously (Shen *et al.*, 2006; Wu *et al.*, 2009; Shang *et al.*, 2010). Mature rosette leaves of 4-week-old plants were used for the stomatal aperture assay, which was conducted under normal air. To assay ABA-induced stomatal closure, leaves were immersed in a solution containing 50 mM KCl and 10 mM MES-KOH (pH 6.5), and exposed to a halogen cold light source for 3 h. Subsequently, (±)ABA or an equal amount of ethanol for dissolving ABA (as the ABA-free controls) at different concentrations was added into the buffer. Stomatal apertures were measured 2.5 h after ABA treatment. To assay ABA-inhibited stomatal opening, leaves were immersed in the same solution as described above in the dark for 12 h before they were transferred to the cold light for 2.5 h in the presence of ABA, and then apertures were determined. Five plants for each genotype (Col, *pyr1 pyr1 pyl1 pyl2 pyl4* quadruple mutant, and *cch* and *rit1* mutants) and one mature rosette leaf from each plant was sampled for the stomatal aperture assay, and five leaves were used in total for each experiment. More than 20 stomata were measured for each leaf, and so more than 80 stomata were measured for each experiment. The experiment was conducted line- and treatment-blind, and repeated independently three times with similar results.

**Water loss and drought assays**

For the water loss assay, rosette leaves were detached from the roots and placed on a plastic dish. Water loss was evaluated by weighing excised leaves at the indicated times under room temperature conditions. For drought treatment, plants were grown on soil for ~5 d and then drought was imposed by withdrawing irrigation until the lethal effect of dehydration was observed on the majority of the plants, whereas the other half were grown under a standard irrigation regime as a control.

**Measurement of ROS and NO production**

The production of ROS and NO in guard cells was estimated using the fluorescence indicators 2',7'-dichlorodihydrofluorescein diacetate (H₂DCF-DA) and diaminofluorescein-FM diacetate (DAF-FM-DA) (Sigma-Aldrich, St Louis, MO, USA), respectively. The epidermal strips were pre-incubated for 2 h under conditions promoting stomatal opening in the MES-Tris buffer (pH 6.15; pre-incubation buffer) supplemented with 0 (ethanol, as a control) or 10 μM (±)ABA, and were incubated in buffer containing 50 mM Tris-HCl (pH 7.2) with 50 μM H₂DCF-DA or 20 mM HEPES-NaOH buffer (pH 7.4) with 10 μM DAF-FM-DA in the dark for 20 min. After the treatment, the epidermal tissues were washed with the same pre-incubation buffer to remove excess dye. Examinations of peel fluorescence were performed using a fluorescence microscopy (Zeiss, Oberkochen, Germany; excitation, 488 nm; emission, 525 nm). All pictures were taken under the same exposure intensity to reduce the influence of the background intensities. Image J software was used to calculate the corrected average optical density (OD) to represent fluorescence intensities, which are the result of the guard cell OD minus background OD.

**ColP in plants**

The ColP assay was performed essentially as described previously (Shang *et al.*, 2010). Myc-tagged OST1 over-expression lines were used to perform the ColP assay. The plant total protein was prepared using extraction buffer (3 mg/ml) containing 50 mM Tris-HCl (pH 7.4), 10 % glycerol (v/v), 1 mM EDTA, 150 mM NaCl, 0.1 % Triton X-100 (v/v), 1 mM PMSF, and 5 μg/ml protein inhibitor cocktail (Roche). Total protein was pre-cleared with the protein A/G plus beads (Santa Cruz Biotechnology, Dallas, TX, USA) and divided into two parts; one incubated with mouse anti-Myc-tag antibody (MBL, Nagoya, Japan) and the other incubated with pre-immune serum (MBL, Nagoya, Japan) for 1 h. After incubation, the protein A/G plus beads were added into the buffer and incubation continued at 4 °C for another 4 h. The beads were washed five times extensively with extraction buffer and then resuspended in protein loading buffer. The immuno-precipitates were separated on a 10 % SDS-PAGE and analysed by immunoblotting with anti-ABAR serum. The anti-ABAR serum was produced as described previously (Shen *et al.*, 2006; Wu *et al.*, 2009; Shang *et al.*, 2010).

**Quantitative real-time PCR analysis**

Total RNA was extracted from 2-week-old seedlings with the RNasy plant mini kit (Qiagen, Hilden, Germany) according to the
manufacturer’s instructions. Single-strand cDNA was synthesized by using total RNA (2 μg) with the M-MLV reverse transcriptase (NEB, Ipswich, MA, USA). Quantitative real-time PCR (qRT-PCR) was performed using the CFX96™ Real-Time System of C1000™ Thermal Cycler (Bio-Rad, Hercules, CA, USA) and SYBR Premix Ex Taq (TaKaRa Bio, Dalian, China) with the program: 5 min at 94 °C and then 30 cycles of 5 sec at 94 °C, 30 sec at 60 °C. ACTIN2/8 gene was used as an internal control. Primers for qRT-PCR are listed in Supplementary Table S1. The qRT-PCR was performed in triplicate and means of the three biological repeats were calculated to represent gene expression level.

Phos-tag SDS-PAGE assay to test phosphorylation

SDS-PAGE was performed according to the method of Laemmli (1970). The Phos-tag ligand AAL-107 was purchased from Wako Pure Chemical Industries (Osaka, Japan). Mn²⁺-Phos-tag SDS-PAGE was performed according to manufacturer’s guidebook. The acrylamide pendant Phos-tag ligand with final concentration of 50 μM and two equivalents of MnCl₂ were added into the gel before polymerization. Electrophoresis was performed at 30 mA until the bromophenol blue dye reached the bottom of the separating gel. Immunoblotting was performed according to previously described procedures (Shen et al., 2006; Wu et al., 2009) with anti-His-tag (MBL, Nagoya, Japan) or anti-CHLH/ABAR serum for detecting corresponding target proteins.

To assay the phosphorylation of ABAR, 3-week-old plants of Col and srk2e were treated with ABA-free (-ABA) or ABA-containing solution (50 μM (±)ABA) for 90 min, then the total protein was prepared from these plants using extraction buffer containing 50 mM Tris-HCl (pH 8.0), 5 mM MgCl₂, 0.1 mM MnCl₂, 0.02% Triton X-100 (v/v), 100 μM PMSF, and 5 μg ml⁻¹ protein inhibitor cocktail. The total protein was used for Mn²⁺-Phos-tag SDS-PAGE assay.

To assay the His-tagged phosphorylation of the C-terminal domain of the KAT1 protein, the recombinant truncated KAT1 protein containing the C-terminal region His301–Asn677 was treated with alkaline phosphatase (AP, Sigma-Aldrich, St Louis, MO, USA) in a 50 mM-Tris-HCl buffer (pH 8.5) containing 1 mM MgCl₂, for 6 h at 37 °C, and purified using Ni-NTA beads. After purification, the eluted protein was dialyzed against AP reaction buffer.

The total protein used for the KAT1 phosphorylation was prepared from 3-week-old plants of Col, quadruple, and cch mutants treated with the ABA-free (−ABA) or ABA-containing solution (50 μM (±)ABA) for 90 min. The buffer used for extracting the total protein contained 50 mM Tris-HCl (pH 8.0), 1 mM MgCl₂, 0.1 mM MnCl₂, 1 mM NaF, 0.02% TritonX-100 (v/v), and 5 μg ml⁻¹ protein inhibitor cocktail. The total protein (30 μg) from the different genotypes was incubated in the medium containing the purified AP treatment KAT1801–677 protein (as a substrate, 2 μg) in the presence of 50 μM ATP for 3 h at room temperature. The reaction mixture was analysed by Mn²⁺-Phos-tag SDS-PAGE assay.

Results

**ABAR interacts with OST1**

To identify the interaction partners of ABAR, a fragment encoding the C-terminus of ABAR (aa 692–1381, ABAR<sub>690</sub>) was used as a bait to screen the *Arabidopsis* cDNA library in a yeast two-hybrid system, and OST1 was identified as a candidate. Further assays were performed to confirm the interaction between ABAR and OST1. Full-length OST1 was cloned to pGADT7 fused with the AD, and the ABAR fragment encoding ABAR<sub>690</sub> was cloned into pGBKKT7 fused with the BD. The yeast cells co-transformed with the construct pair AD-OST1 plus BD-ABAR<sub>690</sub> or BD-53 plus AD-T (a positive control) were able to grow in the SD4-drop-out medium (lacking Leu, Trp, His, and Ade) and turned blue in the presence of α-Gal (Fig. 1A), while the yeast cells co-expressing the construct pairs AD plus BD-ABAR<sub>690</sub> and BD plus AD-OST1, taken as negative controls, were not able to grow in the SD4-drop-out medium (Fig. 1A), indicating that ABAR interacts with OST1 and that the interaction detected in this yeast system is specific and reliable. Co-IP assays in the yeast cells confirmed the interaction of ABAR with OST1 in the yeast system (Fig. 1B). The further experiments showed that, whereas ABAR<sub>690</sub>—the C-terminal half of ABAR—is an interaction domain, neither the N-terminal region of ABAR (aa 1–691, ABAR<sub>691</sub>) nor the middle section of ABAR (aa 692–941, ABAR<sub>690</sub>) interacts with OST1 (Fig. 1C). The interaction of the C-terminal half of ABAR with OST1 was further confirmed in a pull down assay with the recombinant C-terminal half of ABAR and OST1 proteins (Fig. 1D), consistent with the idea that ABAR interacts with OST1 in the cytosolic space through its C-terminal half.

Neither mutation nor over-expression of ABAR gene significantly changes ABA-insensitive phenotypes of the OST1 knockout mutant srk2e

Next, it was tested whether ABAR and OST1—two positive regulators in guard cell signalling in response to ABA (Mustilli et al., 2002; Yoshida et al., 2002; Shen et al., 2006; Legnaioli et al., 2009; Wu et al., 2009; Jia et al., 2011; Tsuzuki et al., 2011, 2013; Du et al., 2012; Zhang et al., 2013)—functionally interact. It was observed that stomatal apertures of both the cch single and srk2e cch double mutants were slightly larger than that of wild-type Col plants under the exogenous ABA-free conditions, but the differences were not always statistically significant. This seems to be the result of the nuance of changes in environmental conditions among the independent repetitions, which might cause differences in guard cell
The intensity of the ABA-insensitive phenotypes of the srk2e cch double mutant in ABA-induced stomatal closure and ABA-inhibited stomatal opening was shown to be comparable with that of both cch and srk2e single mutants with 25 μM (±)ABA application, while in a higher ABA concentration [50 μM (±)ABA], this ABA-insensitive intensity of the srk2e cch double mutant was stronger than that of the cch single mutant and remained similar to that of the srk2e single mutant (Fig. 2A). The detached leaves of the three mutant plants lost water faster than those of wild-type Col plants, where the double mutant srk2e cch showed the highest loss rate, followed by srk2e and cch (Fig. 2B, C). The sensitivities to drought of these mutants showed similar trends to the water loss rates of their detached leaves (Fig. 2D).
The observations of the dehydration assays with both the detached leaves and whole plants are consistent with those of stomatal movement. It has been known that the over-expression of either the C-terminal half of ABAR (aa 631–1381) in whole Col plants (ABAR\textsubscript{631–1381} OE, Wu et al., 2009) or the full-length ABAR specifically in guard cells (Tsuzuki et al., 2013) confers ABA hypersensitivity in ABA-mediated stomatal response. ABAR\textsubscript{631–1381} - over-expression lines were created under the srk2e mutant background by crossing (ABAR\textsubscript{631–1381} OE/srk2e, Supplementary Fig. S2), which did not suppress the srk2e mutant phenotype, but showed an ABA-insensitive phenotype, like the srk2e background, in ABA-induced stomatal closure and ABA-inhibited stomatal opening (Fig. 3A). In addition, whereas over-expression of ABAR\textsubscript{631–1381} in the Col background improved dehydration tolerance, over-expression of the same truncated ABAR under srk2e mutant did not affect the dehydration overly sensitive phenotypes of the srk2e mutant (Fig. 3B–D), which is consistent with the data from the investigation on stomatal movement in response to ABA (Fig. 3A).

Over-expression of OST1 suppresses ABA-insensitive phenotypes of the ABAR mutant cch

To further investigate functional interaction between ABAR and OST1, OST1-over-expression lines were generated in which the OST1 protein was Myc-tagged (Supplementary Fig. S3A). The OST1-transgenic lines displayed ABA-hypersensitive response in stomatal movement as previously reported (Acharya et al., 2013), and the intensities of the ABA-hypersensitive phenotypes were positively correlated with the OST1-expression levels (Supplementary Fig. S3B). The OST1 over-expression line (OST1OE-1) was crossed with the cch mutant to create an OST1 over-expression line under the cch mutant background (OST1OE-1/cch). This OST1OE-1/cch line showed ABA-hypersensitive phenotypes in ABA-induced stomatal closure and ABA-inhibited stomatal opening like the OST1 over-expression line, which suppresses ABA-insensitive phenotypes of the cch mutant (Fig. 4A). The OST1OE-1 showed dehydration tolerance in contrast to cch that is dehydration hypersensitive, and the OST1OE-1/cch line showed dehydration tolerance like the OST1OE-1 line in the assays in both detached leaves and whole plants (Fig. 4B–D), which is consistent with the data from the assays of stomatal movement in response to ABA (Fig. 4A).

Both cch and rtl1 mutations in the ABAR gene impair ABA-induced ROS and NO production like the pyl1 pyl2 pyl4 quadruple mutant

To assess a possible mechanism by which ABAR and OST1 interact in ABA signalling, ABA-induced ROS and NO
production in guard cells was measured in the ABAR gene mutants, using the pyr1 pyl1 pyl2 pyl4 quadruple mutant of the PYR/PYL/RCAR gene family, encoding the cytosolic receptors for ABA upstream of OST1 as a control. It was first confirmed that the two mutant alleles, cch and rtl1, of the ABAR gene show both strong ABA-insensitive phenotypes in ABA-induced stomatal closure and inhibition of light-induced stomatal opening, while the pyr1 pyl1 pyl2 pyl4 quadruple mutant shows strong ABA-insensitive phenotypes only in ABA-induced stomatal closure but not in ABA inhibition of light-induced stomatal opening (Fig. 5). The pyr1 pyl1 pyl2 pyl4 quadruple mutant showed slight ABA-insensitive phenotypes in ABA inhibition of stomatal opening with 15 μM (±)ABA application, and the phenotypic intensity was significantly weaker than that of cch or rtl1, while in a higher ABA concentration [30 μM (±)ABA], the pyr1 pyl1 pyl2 pyl4 quadruple mutant showed a wild-type ABA response in light-induced stomatal opening (Fig. 5), which is largely consistent with previous observations (Yin et al., 2013).

It is known that the pyr1 pyl1 pyl2 pyl4 quadruple mutant shows ABA insensitivity in ABA-induced ROS and NO production (Yin et al., 2013). It was observed that ABA-induced ROS and NO production was impaired in guard cells of cch and rtl1 mutants, which was similar to that in the pyr1 pyl1 pyl2 pyl4 quadruple mutant (Fig. 6A–D). The expression of some ROS metabolism-related genes was further tested. RbohD and RbohF encode two members of the NADPH oxidase family, which promote ROS production and are involved in ABA signalling likely downstream of OST1 (Kwak et al., 2003; Acharya et al., 2013). GPX1/2/5/6/7 encode five members of the glutathione peroxidase family, of which the expression is induced by environmental stresses (Sugimoto and Sakamoto, 1997; Rodriguez et al., 2003); CAT1/2/3 encode three members of the catalase family (Chevalier et al., 1992). The GPXs and CATs are responsible for ROS scavenging (Noctor et al., 2002; Smykowski et al., 2010). These enzymes are involved in maintaining ROS homeostasis in plant cells (Wang and Song, 2008). It was observed that ABA-induced expression of all these genes except for RbohD and CAT3 significantly decreased in both the pyr1 pyl1 pyl2 pyl4 and cch mutants (Fig. 6E–F), suggesting that both ROS production and scavenging processes are impaired in response to ABA in these mutants. These findings are consistent with the above-mentioned decline in the ABA sensitivity of ROS production of these mutants. Together, all the data suggest that CHLH/ABAR, like the PYR/PYL/
RCAR receptors for ABA, acts upstream of ROS and NO in the ABA signalling pathway.

It was further tested, in the yeast one-hybrid system, whether the two important ABA-responsive transcription factors acting downstream of OST1, ABF4, and ABI5, may possibly bind the promoters of the ROS-metabolism-related genes to regulate their expression and ROS homeostasis. The results showed that neither ABF4 nor ABI5 binds to the promoter of *RbohD*, *RbohF*, *GPX1*, *GPX2*, *GPX5*, and *CAT2*, and seems to be unlikely to bind to the promoters of *CAT1* and *CAT3* (Supplementary Fig. S4). OST1 and ABAR did not associate with these promoters either, likely because they are not transcription factors (Supplementary Fig. S4). These data suggest that OST1 may not regulate ROS homeostasis downstream of ABAR and PYR/PYL/RCAR through ABA-responsive transcription factors such as ABF4 and ABI5, but is likely to regulate ROS-metabolism-related enzymes through direct phosphorylation at the post-translational level (Sirichandra et al., 2009; Acharya et al., 2013). It is not precluded, however, that OST1 phosphorylates transcription factors other than ABF4 and ABI5 to regulate ROS-metabolism-related gene expression, which needs further study.

**Phosphorylation of ABAR is independent of OST1 and ABA**

Upon activation by ABA, OST1 modulates the activities of downstream effectors to regulate stomatal movement by phosphorylation (Sato et al., 2009; Sirichandra et al., 2009; Geiger et al., 2009, 2010; Lee et al., 2009, 2013; Brandt et al., 2012; Acharya et al., 2013; Imes et al., 2013; Osakabe et al., 2013; Liang and Zhang, 2014). A recent report suggests that ABAR may be phosphorylated (Wang et al., 2013a). It was tested whether ABAR is a substrate of OST1. In the Phos-tag SDS-PAGE assay, in which the phosphorylated proteins with the phosphate group bound to the divalent metal ions decreases the migration speed, separated ABAR bands were observed on the gels (Fig.7A), indicating that ABAR was phosphorylated (Wang et al., 2013a). However, the amount of phosphorylated ABAR in wild-type Col plants was comparable with that in the *srk2e* mutant, and ABA treatment did not change the amount of phosphorylated ABAR in wild-type Col plants or in the *srk2e* mutant (Fig. 7A), suggesting that phosphorylation of ABAR is independent of OST1 and ABA.
phosphatase-treated KAT1
to KAT1 phosphorylation assays in total proteins prepared from different genotypes. The KAT1 phosphorylation activity was shown to be enhanced by ABA (Fig. 7C), which is consistent with the idea that KAT1 is phosphorylated by the ABA-activated OST1 kinase (Mustilli et al., 2002; Yoshida et al., 2002, 2006; Belin et al., 2006; Fujii and Zhu, 2009; Sato et al., 2009; Acharya et al., 2013). This ABA-induced activation of KAT1 phosphorylation was observed in all the genotypes including wild-type Col, cch, and pyr1 pyl1 pyl2 pyl4 quadruple mutants, of which the levels, however, significantly decreased in both the pyr1 pyl1 pyl2 pyl4 and cch mutants (Fig. 7C).

Discussion

OST1 interacts with, and functions downstream of, ABAR in guard cell signalling in response to ABA

A combination of yeast two-hybrid system, pull down, LCI, CoIP, and SPR assays showed consistently that ABAR interacts directly with OST1 (Fig. 1), a critical signalling component in the PYR/PYL/RCAR-mediated ABA signalling pathway in guard cells (Mustilli et al., 2002; Yoshida et al., 2002; Sato et al., 2009; Sirichandra et al., 2009; Brandt et al., 2012; Acharya et al., 2013; Imes et al., 2013; Osakabe et al., 2013). Although ABAR/CHLH is a chloroplast protein, it spans the chloroplast envelope with its N and C termini exposed to the cytosol (Shang et al., 2010). The C-terminus of ABAR binds to a group of WRKY-domain transcription repressors to regulate expression of ABA-responsive genes (Shang et al., 2010; Liu et al., 2013; Yan et al., 2013). OST1, localized to cytosolic and nuclear spaces (Nakashima et al., 2009; Sirichandra et al., 2010; Ding et al., 2015), interacts with the C-terminal half, but not N-terminal half or middle section of ABAR (Fig. 1). This suggests that the interaction between ABAR and OST1 is likely to take place in the cytosol, which is similar to that between ABAR and the WRKY transcription factors (Shang et al., 2010). However, the cytosolic localization of the interaction between ABAR and OST1 should be confirmed in the future using other techniques, such as bimolecular fluorescence complementation system in Arabidopsis protoplasts.

Neither mutation nor over-expression of the ABAR gene affects significantly ABA-insensitive phenotypes of stomatal movement in the OST1 knockout mutant allele srk2e. However, over-expression of the OST1 gene suppresses ABA-insensitive phenotypes of the ABAR mutant allele cch in stomatal movement (Figs 2–4). These genetic data demonstrate that OST1 functionally interacts with, and acts downstream of, ABAR in ABA signalling in guard cells. In addition, ABAR protein is shown to be phosphorylated, but independently of the OST1 protein kinase, which is consistent with the idea that ABAR functions upstream of OST1 (Fig. 7). These genetic and biochemical findings allow a functional link between ABAR and OST1 to be established in guard cell signalling in response to ABA.
How does ABAR functionally interact with OST1 in ABA signalling in guard cells?

Owing to technical difficulties, the phosphorylation or kinase activity of OST1 when the function of ABAR is lesioned in *cch* or *rtl1* mutants was not determined; however, it is important to understand the functional interaction between the two proteins and this needs to be tested with improved techniques in the future. However, this study has provided several lines of evidence supporting that ABAR, functioning upstream of OST1, shares, at least partly, downstream signalling components with the PYR/PYL/RCAR receptors in guard cells. Whereas the responses of ABA to induce stomatal closure and to inhibit stomatal opening are impaired in two ABAR mutant alleles, *cch* and *rtl1*, only the ABA response to induce stomatal closure was impaired in the *pyr1 pyl1 pyl2 pyl4* quadruple mutant that shows weak to wild-type response in ABA-inhibited stomatal opening (Fig. 5). This phenomenon in the *pyr1 pyl1 pyl2 pyl4* quadruple mutant was previously observed and explained by functional redundancy of the PYR/PYL/RCAR family proteins (consisting of 14 members) or existence of other receptors involved in this ABA response in ABA-inhibited stomatal opening (Yin *et al.*, 2013). Nevertheless, similar to the *pyr1 pyl1 pyl2 pyl4* quadruple mutant, the *cch* and *rtl1* mutants show lesioned ABA responses in ABA-induced ROS and NO production, as well as in ABA-activated phosphorylation of a K⁺ inward channel KAT1 in guard cells (Figs 6 and 7), supporting the idea that ABAR shares these downstream signalling regulators...
with PYR/PYL/RCAR receptors in guard cell signalling. Therefore, ABAR functions to directly interact with OST1 to regulate downstream signalling components such as ROS, NO, and KAT1 in a mechanism similar to the PYR/PYL/RCAR-mediated ABA signalling pathway in guard cells where PYR/PYL/RCAR receptors regulate OST1 through clade A PP2Cs to interact with ROS and NO messengers to modulate the function of the inward K⁺ channels such as KAT1 (Pei et al., 2000; Zhang et al., 2001; Mustilli et al., 2002; Neill et al., 2002; Garcia-Mata et al., 2003; Kwak et al., 2003; Bright et al., 2006; Acharya et al., 2013; Wang et al., 2015).

In addition, it was previously reported that ABA inhibits BL-mediated stomatal opening in part via ABA-activated guard cell H⁺-ATPase phosphorylation mediated by OST1 (Hayashi and Kinoshita, 2011; Hayashi et al., 2011), and ABAR/CHLH regulates guard cell H⁺-ATPase phosphorylation, which may be a mechanism to explain the role of ABAR in regulating ABA-induced inhibition of BL-induced stomatal opening (Tsuzuki et al., 2013). In this regard, ABAR is likely to modulate H⁺-ATPase phosphorylation through OST1 in guard cells, which may be a key process to regulate inward ion flux across the plasma membrane of guard cells to affect stomatal opening. Further investigations will be needed to elucidate cooperation or crosstalk of ABAR-mediated signalling with PYR/PYL/RCAR-mediated signalling, in which the genetic interactions between ABAR and PYR/PYL/RCAR in guard cell

Fig. 7. Phosphorylation assessment of the ABAR and KAT1 proteins by Phos-tag SDS-PAGE test. (A) ABAR phosphorylation in the srk2e mutant, detected with ABAR-immunoblot assay using Phos-tag SDS-PAGE test. Three-week-old plants of wild-type Col or srk2e mutant were treated with ABA-free (−ABA) or ABA-containing solution (+ABA) [50 μM (+)ABA] for 90 min, then ABAR phosphorylation status was detected with anti-ABAR serum (+Phos-tag, top panel). Black and open arrows indicate the phosphorylated and dephosphorylated forms of ABAR, respectively. -Phos-tag SDS-PAGE (bottom panel) shows a loading control and indicates the ABAR protein with the expected molecular mass of ABAR (about 150 kDa). The experiment was replicated five times with similar results. (B) Phosphorylation level of the His-tagged C-terminal domain of KAT1. The recombinant truncated KAT1 containing C-terminal region His301–Asn677 (KAT1301–677) was treated with alkaline phosphatase-containing (+AP) or AP-free buffer (−AP) for 6 h. The phosphorylation of KAT1301–677 was assayed by a slower mobility in a Phos-tag SDS-PAGE that selectively retards phospho-KAT1301–677 (black arrow), where the His-tagged KAT1301–677 was detected by anti-His-Tag serum. Note that the AP treatment increases the amount of the dephosphorylation form of KAT1301–677 (open arrow). The bottom panel (indicated by -Phos-tag) shows a loading control. (C) ABA-activated phosphorylation of the KAT1301–677 protein in wild-type Col, quadruple, and cch mutant plants. Three-week-old plants were treated with ABA-free (−ABA) or 50 μM (+)ABA-containing medium (+ABA) for 90 min. The phosphorylation was assayed in the extracted total protein, using KAT1301–677 as a substrate, and the His-tagged KAT1301–677 was detected by anti-His-Tag serum (top panel). Black and open arrows indicate phosphorylated and dephosphorylated KAT1301–677, respectively. Note that the amounts of phosphorylated KAT1301–677 protein are apparently comparable among the three genotypes Col, quadruple, and cch with ABA-free treatment (−ABA). The amounts of the phosphorylated and dephosphorylated KAT1301–677 protein with ABA treatment were evaluated by scanning the protein bands, and relative band intensities, normalized relative to the intensity of the ABA-free treatment sample (as 100%), are indicated by numbers above the bands. Note that, in comparison with Col, the levels of the ABA-activated phosphorylated KAT1301–677 protein in quadruple and cch decrease. -Phos-tag SDS-PAGE (bottom) shows a loading control and indicates the expected molecular mass of His-tagged KAT1301–677 (about 60 kDa). The experiment was repeated five times with similar results.
signalling in response to ABA, for example, need to be determined in the future. These studies will help to understand the complex ABA signalling network.

**Supplementary data**

Supplementary data are available at *JXB* online.

**Fig. S1.** Interaction of ABAR with OST1 tested with the SPR system.

**Fig. S2.** Identification of the GFP-tagged ABAR expression in the srk2e mutant plants.

**Fig. S3.** Phenotypes of the Myc-tagged OST1-over-expression lines.

**Fig. S4.** Yeast one-hybrid assays to test possible interactions of ABF4, AB15, OST1, or ABAR with the promoters of ROS-metabolism-related genes *RbohD*, *RbohF*, *GPX1*, *GPX2*, *GPX5*, *CAT1*, *CAT2*, and *CAT3*.

**Fig. S5.** Purified recombinant truncated KAT1 protein (*KAT1* 1301–677, aa 301–677) tested with an SDS-PAGE gel.

**Table S1.** PCR primers used in this study.

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