Journal of Experimental Botany, Vol. 63, No. 2, pp. 1013–1024, 2012
doi:10.1093/jxb/err338 Advance Access publication 9 November, 2011

Journal of Experimental Botany
www.jxb.oxfordjournals.org

RESEARCH PAPER

A rice orthologue of the ABA receptor, OsPYL/RCAR5, is a positive regulator of the ABA signal transduction pathway in seed germination and early seedling growth

Hyunmi Kim[1], Hyunsik Hwang[1], Jung-Woo Hong[2], Young-Na Lee[1], Il Pyung Ahn[1], In Sun Yoon[1], Sang-Dong Yoo[2], Sukchan Lee[3], Sung Chul Lee[4,*] and Beom-Gi Kim[1,*]

[1] Department of Bio-crop development, National Academy of Agricultural Science, Rural Development Administration, Suwon, 441-707 Korea
[2] Department of Biological Sciences, Sungkyunkwan University, Suwon, 440-746 Korea
[3] Department of Genetic Engineering, Sungkyunkwan University, Suwon, 440-746 Korea
[4] School of Biological Sciences (BK21 program), Chung-Ang University, Seoul, 156-756 Korea

* To whom correspondence should be addressed. E-mail: bgkimpeace@gmail.com or sclee1972@cau.ac.kr

Received 20 August 2011; Revised 26 September 2011; Accepted 26 September 2011

Abstract

Abscisic acid (ABA) is a phytohormone that positively regulates seed dormancy and stress tolerance. PYL/RCARs were identified an intracellular ABA receptors regulating ABA-dependent gene expression in *Arabidopsis thaliana*. However, their function in monocot species has not been characterized yet. Herein, it is demonstrated that PYL/RCAR orthologues in *Oryza sativa* function as a positive regulator of the ABA signal transduction pathway. Transgenic rice plants expressing OsPYL/RCAR5, a PYL/RCAR orthologue of rice, were found to be hypersensitive to ABA during seed germination and early seedling growth. A rice ABA signalling unit composed of OsPYL/RCAR5, OsPP2C30, SAPK2, and OREB1 for ABA-dependent gene regulation was further identified, via interaction assays and a transient gene expression assay. Thus, a core signalling unit for ABA-responsive gene expression modulating seed germination and early seedling growth in rice has been unravelled. This study provides substantial contributions toward understanding the ABA signal transduction pathway in rice.

Key words: ABA receptor, PP2C, rice, SnRK2.

Introduction

The phytohormone abscisic acid (ABA) is a multifunctional plant hormone which plays a role in several different physiological processes including seed dormancy, abiotic stress tolerance, senescence, and developmental differentiation of plants. In particular, it has been characterized as a stress hormone because it is an important endogenous small molecule that mediates stress responses, including stress-responsive gene expression, stomatal closure, and vegetative growth modulation (Adie *et al.*, 2007; McCourt and Creelman, 2008; Rodriguez-Gacio Mdel *et al.*, 2009). Thus, a great deal of effort has been focused on elucidating the molecular mechanisms underlying ABA sensing and signalling over the past few decades (Umezawa *et al.*, 2010). For example, ABA-dependent gene expression mechanisms have been particularly well characterized with respect to key signalling kinases and phosphatases that connect the upstream signalling perception to downstream gene responses in *Arabidopsis* (Kuhn *et al.*, 2006; Nakashima *et al.*, 2009; Sirichandra *et al.*, 2010). In the absence of ABA, clade A protein phosphatase 2Cs (PP2Cs) including ABSCISIC ACID INSENSITIVE 1 (ABI1), ABI2, PP2CA/AHG3, AHG1, HAB1, and HAB2 inhibit SnRK2 subclass III kinase activity via dephosphorylation. In the presence of ABA, the PP2Cs are inactivated, and autophosphorylated SnRK2s become active (Umezawa *et al.*, 2009). Activated SnRK2s phosphorylate and activate ABFs, which are bZIP

transcription factors that bind to ABA-responsive elements (ABREs). As a consequence, active ABFs promote the expression of ABA-responsive genes such as RD29 and RAB16 (Kagaya *et al.*, 2002; Sirichandra *et al.*, 2010). However, how ABA is perceived and involved in the inactivation of clade A PP2Cs have represented missing pieces of the puzzle in the ABA signalling pathway.

Recently, two independent research groups discovered novel intracellular ABA receptors, PYL/RCARs, that are involved in ABA sensing and signalling via their direct interaction with clade A PP2Cs in *Arabidopsis thaliana*. One group used yeast two-hybrid assays to identify proteins that interact with ABI1, a member of clade A PP2Cs (Ma *et al.*, 2009). These interactors, referred to as RCARs (regulatory components of ABA receptors), were intracellular ABA receptors, according to the results of ABA binding assays and ABA-responsive phenotype assays using gain- and loss-of-function mutants (Ma *et al.*, 2009). Another group isolated the same ABA receptors via map-based cloning of the pyrabactin- (an analogue of ABA) resistant mutant 1 (*pyr1*), and named them as *PYR* and *PYR-like* (*PYL*) (Park *et al.*, 2009; Santiago *et al.*, 2009*b*).

Both research groups demonstrated that ABA-bound PYL/RCARs interact with clade A PP2Cs and inhibits their phosphatase activity, resulting in an activation of subclass III SnRK2s in the presence of ABA. Consequently, ABA signalling is activated and the expression of ABA-responsive genes, such as *RD29* and *RAB16*, is induced. Fujii *et al.* (2009) reconstituted the core components of the ABA signal transduction pathway consisting of PYLs, PP2Cs, SnRK2 subclass III, and ABFs in *Arabidopsis* protoplasts, and determined that these were necessary and sufficient for the ABA-dependent expression of luciferase fused to the *RD29B* promoter. These results showed that PYL/RCARs are a central piece of the puzzle regarding the manner in which ABA is perceived and involved in ABA-responsive gene regulation. After the genetic and biochemical identification of PYL/RCARs as cellular ABA receptors, several groups have determined the protein structure of the complex bewteen PYL/RCARs and PP2Cs via X-ray crystallography (Melcher *et al.*, 2009, 2010; Miyazono *et al.*, 2009; Nishimura *et al.*, 2009; Peterson *et al.*, 2010). ABA binding appears to change the structure of PYL/RCARs and exposes the interface at which clade A PP2Cs bind, and such an interaction locks the ABA-binding pocket of the receptor. In plants, cellular ABA receptor PYL/RCAR orthologues appear to be highly evolutionarily conserved (Klingler *et al.*, 2010; Umezawa *et al.*, 2010). Umezawa *et al.* (2010) demonstrated, on the basis of bioinformatic analyses, that a bryophyte has four ABA receptor homologues but that vascular plants have >10 homologues. Despite their importance, PYL/RCARs in monocot plants have only recently been identified based on sequence homology, but their functions have not been identified yet. In this study, an attempt was made to determine whether the rice PYL/RCAR orthologues were authentic ABA receptors, and to characterize their physiological functions. The hierarchy of interactions among the components of the ABA signal transduction pathway consisting of PYL/RCAR, PP2Cs, SAPKs, and a bZIP transcription factor was also identified. All of these components were subsequently reconstituted in *Arabidopsis* protoplasts in an effort to reconfirm whether this interaction network was necessary and sufficient to induce ABA-dependent gene expression. The understanding of a complete ABA signal transduction pathway in rice will provide useful information to improve the abiotic stress tolerance of crop plants.

Materials and methods

Plant materials, growth conditions, and transformation of rice

Oryza sativa cv Dong-Jin was used in this study. Rice seed surfaces were sterilized by treatment with 70% alcohol for 3 min, treatment with 50% ROX containing Tween-20 for 20 min, treatment with 50% ROX for 20 min, and eight washes with distilled water. These seeds were grown on half-strength Murashige and Skoog (MS) medium (supplemented with 1% sucrose, 0.4% phytagel and adjusted to pH 5.8) for 2 weeks under long-day conditions (16 h light and 8 h darkness). The coding sequence of the *OsPYL/RCAR5* gene was amplified via PCR from first-strand cDNA. The PCR product was inserted into pENTR/D-Topo (Invitrogen) and then transferred to a pGA2897 vector via an LR recombination reaction of the Gateway system. The resultant Ubi::*OsPYL/RCAR5* construct, in which *OsPYL/RCAR5* is fused to the constitutive maize ubiquitin promoter, was transformed into *Agrobacterium tumefaciens* LBA4404 via electroporation. Rice transgenic plants were generated by the *Agrobacterium*-mediated co-cultivation method and the transformants were selected based on hygromycin resistance and subsequently grown in a greenhouse (Toki *et al.*, 2006).

Germination assay and post-germination assay

Surface-sterilized dehulled seeds were planted on half-strength MS medium supplemented with 0, 2, and 5 μM ABA (2-*cis*-4-*trans*-abscisic acid, 98% synthetic, Sigma-Aldrich). Seeds that formed green shoots were scored as germinated. Seed germination was scored every 12 h for 5 d. Germinated seedlings were then transferred to half-strength MS medium containing hygromycin (40 mg l^{-1}) to determine whether they came from transgenic seeds after germination. Germination rates were measured by counting only transgenic seeds.

For post-germination assays, surface-sterilized dehulled seeds were planted on half-strength MS medium containing hygromycin (40 mg l^{-1}). Five days after planting, similarly grown transgenic plants were transferred to half-strength MS medium supplemented with 0, 2, 5, or 10 μM ABA in square Petri dishes (125×25×20 mm) and grown vertically. Seedling growth was measured 7 d after the transfer.

RT-PCR and quantitative PCR

Total RNA was isolated from 2-week-old seedlings using the RNeasy Plant Mini Kit (Qiagen). Contaminating genomic DNA was digested using recombinant DNase I (RNase-free) (Takara Bio Inc. Ohysu, Japan). From total RNA (5 μg), first-strand cDNA was synthesized with oligo(dT) and random hexamers using the Superscript Reverse Transcriptase first-strand synthesis system (Invitrogen). RT-PCR and quantitative PCR were carried out with internal primers and normalized based on the expression of *Ubi5*, and relative gene expression was analysed via the ΔΔCt method (see Supplementary Table S1 available at *JXB* online).

Yeast two-hybrid assay

Yeast two-hybrid assays were conducted using the Matchmaker™ GAL4 two-hybrid system 3 (Clontech). The PCR products were cloned into the pGEM T-easy vector (Promega), digested with restriction enzymes, and cloned into yeast two-hybrid vectors. OsPP2Cs and OREB1 were cloned into pGADT7, resulting in fusion to the GAL4 activation domain. OsPYL/RCARs and SAPKs were cloned into pGBKT7, resulting in fusion to the GAL4 DNA-binding domain. Using the LiOAC method, each pair of constructs was co-transformed into *Saccharomyces cerevisiae* strain AH109 (*MATa, trp1-901, leu2-3, 112, ura3-52, his3-200,gal4*Δ, *gal80*Δ, *LYS2::GAL1UAS-GAL1TATA-HIS3, GAL2UAS-GAL2TATA-ADE2, URA3::MEL1UAS-MEL1TATA-lacZ, MEL1*), and subsequently plated on SD minimal media (Clontech Inc.) without leucine and tryptophan, then transferred to selection medium without leucine, tryptophan, and histidine, supplemented with 1.5 mM 3-AT (3-amino-1, 2, 4-triazole). Growth was assessed 4 d after transfer.

Rice protoplast isolation

Protoplasts were isolated from etiolated young seedlings (Chen *et al.*, 2006). Surface-sterilized rice seeds were grown on half-strength MS (supplemented with 1% sucrose, 0.4% phytagel and adjusted to pH 5.8) under continuous light conditions for 3 d at 28 °C and transferred to 25 °C dark conditions for 10 d to induce etiolation. The etiolated leaves were chopped and dipped in K3 enzyme solution [K3 solution with 0.4 M sucrose, 1.5% cellulase R-10 (Yakult Honsa Co., Ltd, Tokyo, Japan), and 0.3% macerozyme R-10 (Yakult Honsha)] supplemented with ampicillin (100 mg l^{-1}). This was infiltrated via vacuum for 40 min, followed by 3–5 h of incubation on a shaking incubator. The chopped tissue with K3 enzyme solution was then filtered via centrifugation through miracloth for 4 min at 300 *g*. An equal volume of W5 solution (154 mM NaCl, 125 mM $CaCl_2$, 5 mM KCl, and 2 mM MES, adjusted to pH 5.8) was added to the filtered K3 solution and mixed. Pelleted protoplasts were resuspended in suspension medium (0.4 M mannitol, 20 mM $CaCl_2$, and 5 mM MES, adjusted to pH 5.7). A 10–20 μg aliquot of plasmid DNA was added to this protoplast solution, and it was then mixed with a 40% polyethylene glycol (PEG) solution [40% PEG 4000, 0.4 M mannitol, and 100 mM $Ca(NO^3)_2$, adjusted to pH 7.0]. For transfection, the mixture was incubated for 30 min at 25°C in darkness. W3 solution (500 μl, 1 ml, 2 ml, and 5 ml) was added stepwise for dilution of the PEG solution. After centrifugation, the protoplast pellet was resuspended in W5 solution, and then incubated overnight at 25°C in darkness.

Subcellular localization and bimolecular fluorescence complementation (BiFC)

For green fluorescent protein (GFP) fusion protein expression, the PCR products were inserted into pENTR/D-topo and recombined via an LR recombination reaction into the pMDC83 vector. The fusion constructs (10 μg) were employed for the transfection of rice protoplasts. GFP fluorescence was observed using a fluoview FV300 CLSM system (Olympus Ltd, London, UK) 1 d after transfection.

For BiFC assays, the PCR products for *OsPYL/RCAR5, OsPP2C30*, and *OREB1* were cloned into pSPYNE(R)173, resulting in fusion with the N-terminal portion of the yellow fluorescent protein (YFP) sequence. Os*PP2C30*, *SAPK2*, and *SAPK4* PCR products were cloned into pSPYCE(M), resulting in fusion with the C-terminal portion of the YFP sequence (Waadt *et al.*, 2008). Rice protoplasts were transfected with various pairs of plasmid DNAs (10–20 μg). GFP fluorescence was evaluated using a Fluoview FV300 CLSM system (Olympus Ltd) 1 d after transfection. For transient expression, *A. tumefaciens* strain GV3101 harbouring each construct was employed along with the p19 strain for the infiltration of 6-week-old *Nicotiana benthamiana* leaves. For microscopic analyses, leaf discs were cut 4 d after infiltration. The lower epidermis cells were analysed under confocal microscopy (model Zeiss 510 UV/Vis Meta) using LSM Image Browser software.

Transient gene expression in Arabidopsis thaliana *protoplast*

Arabidopsis protoplast isolation and transient expression assays were followed as previously described (Yoo *et al.*, 2007). Plants were grown on soil under 14 h light and 10 h dark conditions at 23 °C for 4 weeks. Freshly isolated leaf mesophyll protoplasts were co-transfected with the designated effector plasmids and *ABRE promoter::firefly luciferase* reporter plasmids. A *UBQ10 promoter::renilla luciferase* plasmid was employed as a transfection control. Reporter activities were measured using a dual luciferase assay system in accordance with the manufacturer's instructions (Promega). For evaluations of ABA response, 10 μM ABA (Sigma) was applied 1 h after transfection, and then additionally incubated for 5 h at room temperature.

Protein expression was monitored via protein blot analysis using commercially available horseradish peroxidase (HRP)-conjugated antibodies raised against haemagglutinin (Roche) and flag (Sigma) epitopes.

Results

Rice orthologues of Arabidopsis *ABA receptors, PYL/RCARs, are highly conserved and classified into three clades*

In order to isolate PYL/RCAR orthologues from rice, the rice protein database was searched using 14 *Arabidopsis* PYL/RCAR amino acid sequences as queries at the Rice Genome Annotation site (http://rice.plantbiology.msu.edu/blast.shtml). Thirteen ABA receptor candidates that showed e-values $<e^{-10}$ in BLASTP analysis were found. These genes were aligned using Clustal W, and the motifs and amino acid residues that are highly conserved in *Arabidopsis* counterparts were subjected to comparative analysis. Completely conserved residues in both *Arabidopsis* and rice proteins were highlighted in black using the GeneDoc program. Os02g15620 and Os06g33490 were found to have deletions in the N-terminal region, and Os06g33480 has deletions in the C-terminal region. Thus, it appears likely that those genes are not functional ABA receptors, and that there are, at most, 10 functional PYL/RCAR orthologues in rice. The putative rice ABA receptors were named OsPYL/RCARs with suffix numbers. In order to compare genetic diversity among the proteins, a phylogenetic tree of the rice and *Arabidiopsis* PYL/RCARs was built using the Neighbor–Joining method in the MEGA 4.0 software package. The phylogenic tree showed that these families could be categorized into three subgroups (see Supplementary Fig. S1 at *JXB* online).

Constitutive expression of OsPYL/RCAR5 *leads to an ABA-hypersensitive phenotype in seed germination and early seedling growth stages*

In an effort to examine whether the rice orthologues of PYL/RCARs are real functional ABA receptors, a gain-of-function

approach was initially adopted. This may allow gene redundancy issues to be overcome. For example, single and double mutants of *pyr1* and *pyl4* showed no ABA-responsive phenotypes due to gene redundancy. In contrast, the overexpression of *Arabidopsis* RCAR1 or PYL5 exhibited hypersensitivity phenotypes in ABA-related seed germination and root growth inhibition (Ma *et al.*, 2009; Park *et al.*, 2009).

Os05g12260, named *OsPYL/RCAR5*, was selected for functional analysis because it is the highest homology with RCAR1 and it showed clear ABA-dependent interaction with subclass A PP2Cs in yeast two hybridization (Fig. 4A; Supplementary Fig. S1 at *JXB* online). Transgenic rice plants constitutively expressing *OsPYL/RCAR5* were generated. Ten independent transformants were selected in hygromycin medium and further confirmed by genomic PCR of the hygromycin resistance gene (data not shown). The overexpression of *OsPYL/RCAR5* was confirmed by RT-PCR and quantitative PCR. The expression level of *OsPYL/RCAR5* was >40–50 times higher than that of the wild type, showing that the transgenic plants express the *OsPYL/RCAR5* constitutively (Fig. 1A). A germination assay was conducted to monitor the ABA-related phenotype of the transgenic plant lines. All transgenic lines tested showed ABA-hypersensitive phenotypes in germination assays, and three lines were ultimately selected for further study. The transgenic lines showed delayed germination, even in MS medium, and the rate of inhibition of germination was correlated positively with the concentration of ABA in the media, and at 5 μM ABA, the transgenic rice seeds were unable to germinate even after 5 d (Fig. 1B). Post-germination assay results further demonstrated the ABA-hypersensitive phenotype of the transgenic rice lines. After germination, the seedlings were transferred into half-strength MS medium with various concentrations of ABA. The growth of roots and shoots of the *OsPYL/RCAR5*-overexpressing transgenic lines were both inhibited significantly in the MS medium supplemented with ABA compared with growth in MS medium. These ABA-hypersensitive phenotypes indicate that OsPYL/RCAR5 might be a functional orthologue of PYL/RCARs

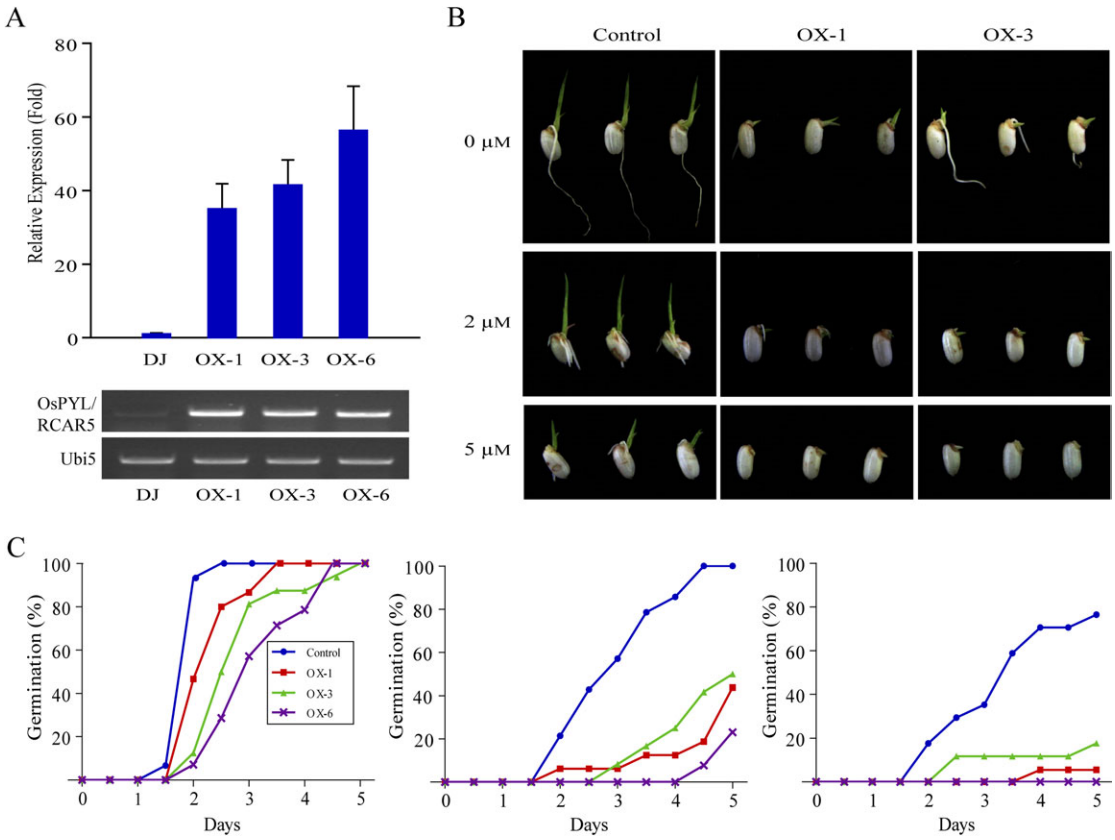

Fig. 1. *OsPYL/RCAR5*-overexpressing transgenic rice showed ABA hypersensitive phenotypes in germination. (A) Quantitative real-time PCR (upper) and RT-PCR (lower) analysis of *OsPYL/RCAR5* expression in *Oryza sativa* cv Dong-Jin (DJ) and Ubi::*OsPYL/RCAR5* transgenic rice lines. Quantitative real-time PCR was analysed via the $2^{-\Delta\Delta CT}$ method using the rice *Ubi5* gene as an internal control. The values are presented as the average and SD from three experiments. RT-PCR was carried out for 25 cycles. Rice *Ubi5* transcripts were amplified as a control (lower gel). (B) Germination inhibition of *OsPYL/RCAR5*-overexpressing (OX) lines via ABA treatment. Seeds of the control (transgenic lines with empty vector) and OX lines were grown on half-strength MS medium containing 0, 2, or 5 μM ABA for 5 d. Photographs were taken on day 5. (C) Germination time courses on half-strength MS medium without ABA (left panel), with 2 μM ABA (middle panel), and with 5 μM ABA (right panel). Three independent experiments were carried out with similar results. Representative graphs are shown (n=20 seeds in each experiment).

that performs a function in seed germination and early seedling growth in rice.

Constitutive expression of OsPYL/RCAR5 *increases expression of the ABA-responsive genes under ABA treatment*

It has been demonstrated that OsPYL/RCAR5 is a positive regulator in ABA signalling via phenotypic analysis of overexpressing transgenic lines (Figs 1, 2). In order to examine whether *OsPYL/RCAR5*-overexpressing transgenic lines could regulate expression of ABA-inducible genes, the expression of ABA-inducible marker genes was monitored by real-time PCR. The rice LEAs (late-embryogenesis abundant) genes such as *RAB16A*, *LEA3*, and *LIP9* are induced by ABA and have ABREs in the promoter region of those genes (Fukao *et al.*, 2011). In the present experiment, those genes were also highly induced under ABA treatment in both the wild type and *OsPYL/RCAR5*-overexpressing transgenic lines, and they were expressed significantly more in *OsPYL/RCAR5*-overexpressing rice than in the wild type. With no ABA treatment, the gene expression of the tested LEA proteins was not induced in either the wild type or *OsPYL/RCAR5*-overexpressing transgenic lines.

OsPYL/RCAR5 interacts with clade A rice PP2Cs in an ABA-dependent manner

In *Arabidopsis*, ABA signalling is mediated by physical protein–protein interaction between PYL/RCARs and clade A PP2Cs. AHG3 of *Arabidopsis* clade A phosphatases was known to function specifically in seed germination and early seedling growth (Kuhn *et al.*, 2006; Yoshida *et al.*, 2006), Rice orthologues of AHG3, Os03g16170, Os05g38290, and Os01g62760, were cloned, named according to nomenclature described by Xue *et al.* (2008), and their gene expression patterns were analysed in three different tissues (see Supplementary Fig. S2 at *JXB* online). All these genes were dominantly expressed in seeds, although they were also

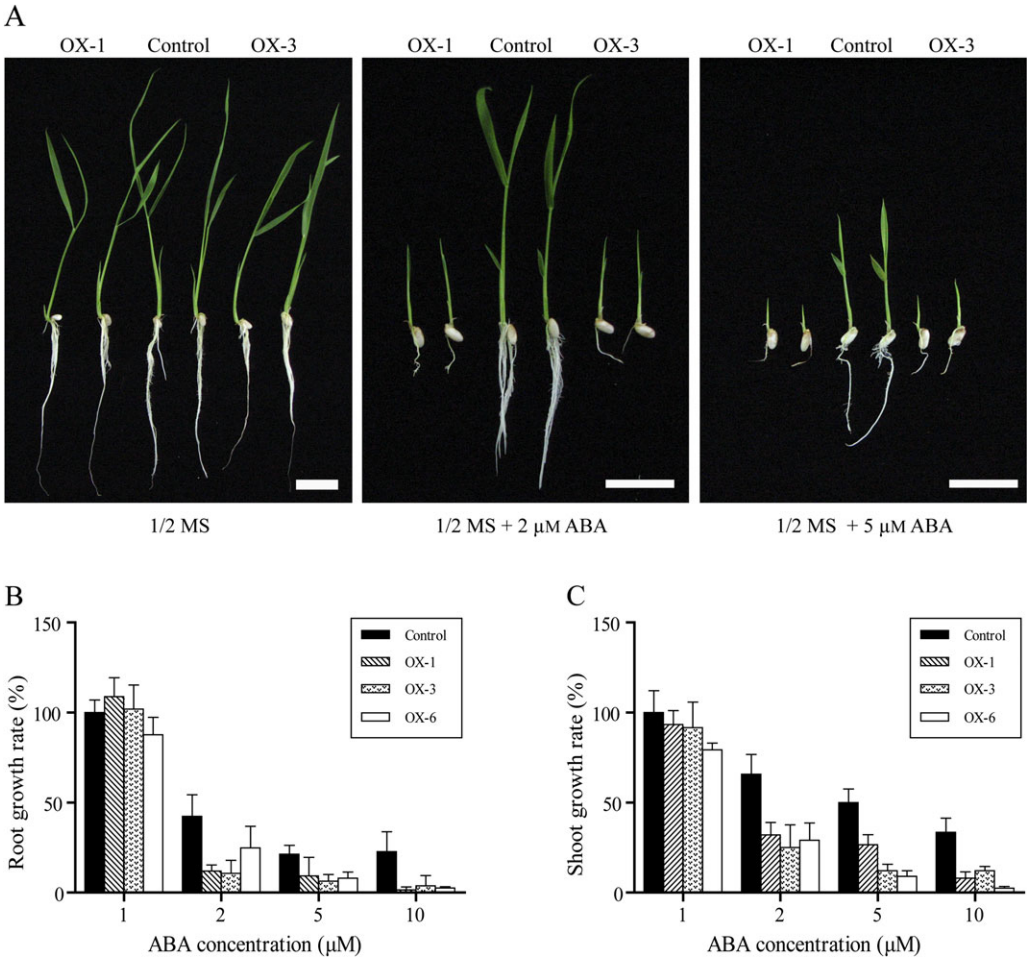

Fig. 2. *OsPYL/RCAR5*-overexpressing rice showed ABA-hypersensitive phenotypes in early seedling growth. Three independent *OsPYL/RCAR5*-overexpressing (OX) lines were transferred to half-strength MS medium supplemented with different ABA concentrations 5 d after germination. (A) Shoot and root growth inhibition of OX lines by ABA treatment. Photographs show representative seedlings 7 d after transfer. (B) Root growth rate of the control (transgenic lines with empty vector) and OX lines. (C) Shoot growth rate of the control (transgenic lines with empty vector) and OX lines. Three independent experiments were carried out with similar results. Representative graphs are presented. Results are presented as means ±SE (n=5 seedlings per experiment). Bar=2 cm.

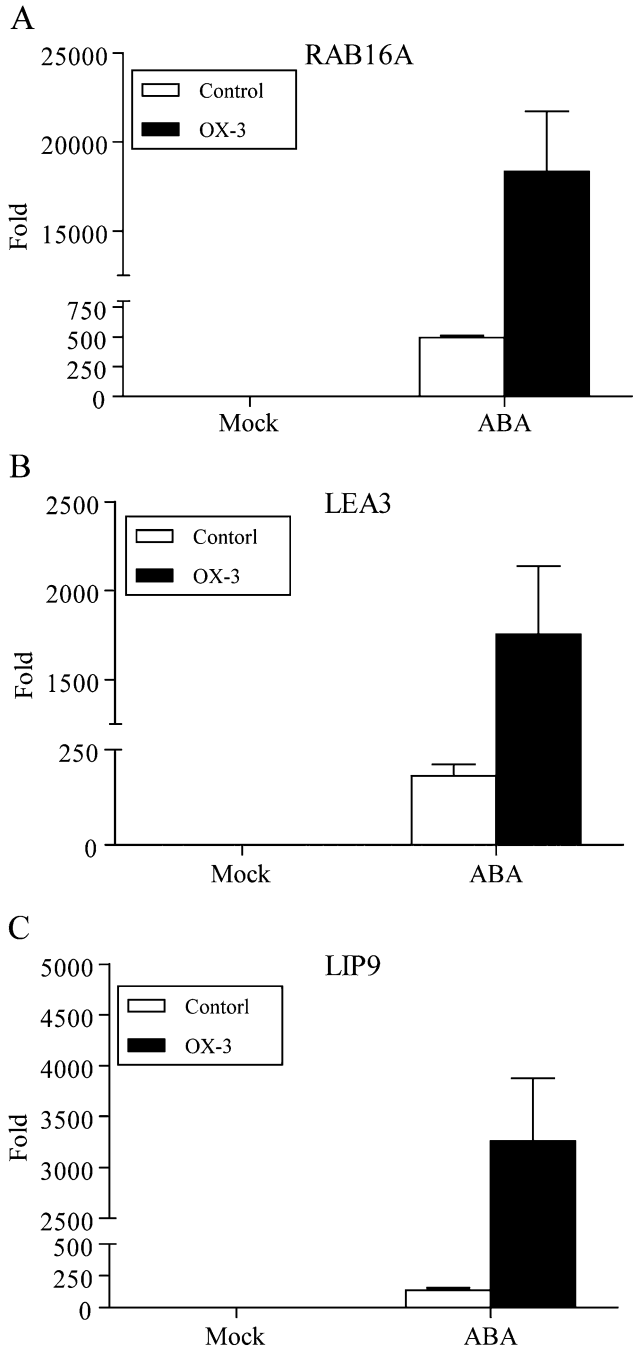

Fig. 3. OsPYL/RCAR5 positively regulate the expression of ABA-responsive genes. Fourteen-day-old wild-type (*Oryza sativar* cultivar Dong-Jin) and Ubi::*OsPYL/RCAR5-3* plants were treated with ABA solution (50 μM in 100% methanol) or mock treated (100% methanol) for 6 h. Shoots were analysed by quantitative RT-PCR (mock-treated Dong-Jin=1.0). Data represent means ±SE from three replicates. (A) RAB16A (LOC_Os11g26790), (B) LEA3 (LOC_Os05g46480), and (C) LIP9 (LOC_Os02g44870) were monitored for gene expression level on ABA treatment.

expressed in other tissues. These genes were also induced by ABA treatment (see Supplementary Fig. S2). In order to determine whether OsPYL/RCAR5 can interact with these OsPP2Cs proteins, the protein–protein interactions between three OsPP2Cs and OsPYL/RCAR5 were analysed using a yeast two-hybrid system. In an effort to confirm the interaction specificity, OsPYL/RCAR3 (Os02g15640), which has the highest homology with OsPYL/RCAR5, was used as a control. In the yeast two-hybrid assay, OsPYL/RCAR3 did not interact with the three PP2CAs. However, OsPYL/RCAR5 interacted with the three OsPP2CAs depending on ABA. (Fig. 4A). In *Arabidopsis*, it has already been reported that PYL/RCARs interact with clade A PP2Cs in an ABA-dependent fashion using the yeast two-hybrid assay (Ma *et al.*, 2009; Park *et al.*, 2009; Santiago *et al.*, 2009*b*). When 10 μM ABA was added to the selection medium, these treatments did indeed affect the interaction between OsPYL/RCAR5 and three OsPP2Cs, with the notable exceptions of OsPYL/RCAR5 and OsPP2C09 (Fig. 4A). In the presence of ABA, but not in its absence, OsPYL/RCAR5 interacted with OsPP2C49 and OsPP2C30. However, OsPP2C09 interacted with OsPYL/RCAR5 independently of ABA. These results provide evidence that OsPYL/RCAR5 might function as an ABA receptor, implying that the ABA-sensing mechanism is conserved in both rice and *Arabidopsis*.

The OsPYL/RCAR5–OsPP2C30 complex is localized to the nucleus

The interaction between OsPYL/RCAR5 and OsPP2C30 *in planta* was further confirmed by BiFC experiments. OsPYL/RCAR5 was cloned into the pSPYNE(R)173 vector, which contain the N-terminal 155 amino acids of YFP, and three OsPP2Cs were cloned into the pSPYCE(M) vector, containing the C-terminal 84 amino acids of YFP. Combinations of BiFC constructs were expressed transiently in *N. benthamiana* leaves via agroinfiltration. The fluorescence was strongly detected in tobacco epidermis cells with OsPYL/RCAR5 and OsPP2C30 (Fig. 4B). The OsPYL/RCAR5–OsPP2C30 complex was clearly localized in the nucleus (Fig. 4B).

The BiFC experiments were then carried out in rice protoplasts in order to confirm the interaction between OsPYL/RCAR5 and OsPP2C30 in its own system. Fluorescence was detected and clearly localized in the nucleus (Fig. 4B, C). It has been determined that both RCAR1–ABI1 and PYL5–HAB1 complexes are localized within the cytoplasm as well as in the nucleus (Ma *et al.*, 2009; Santiago *et al.*, 2009*b*), whereas the FsPP2C1–PYL8 complex is localized specifically in the nucleus; this finding is consistent with the present results (Saavedra *et al.*, 2010). An attempt was also made to clarify the subcellular localization of OsPYL/RCAR5 and OsPP2C30 in the cell via the transient expression of GFP fusion proteins in tobacco epidermis cells and rice protoplasts. OsPYL/RCAR5::GFP was detected in both the cytosol and the nucleus (Fig. 4B, C). In contrast, OsPP2C30::GFP was clearly localized only within the nucleus (Fig. 4B, C). In order to examine whether ABA affects the interaction or localization of OsPYL/RCAR5::GFP, OsPP2C30::GFP, and the OsPYL/RCAR5–OsPP2C30 complex, 10 μM ABA was sprayed onto the agroinfiltrated tobacco leaves or 1 μM ABA was added to the transfected protoplasts. The

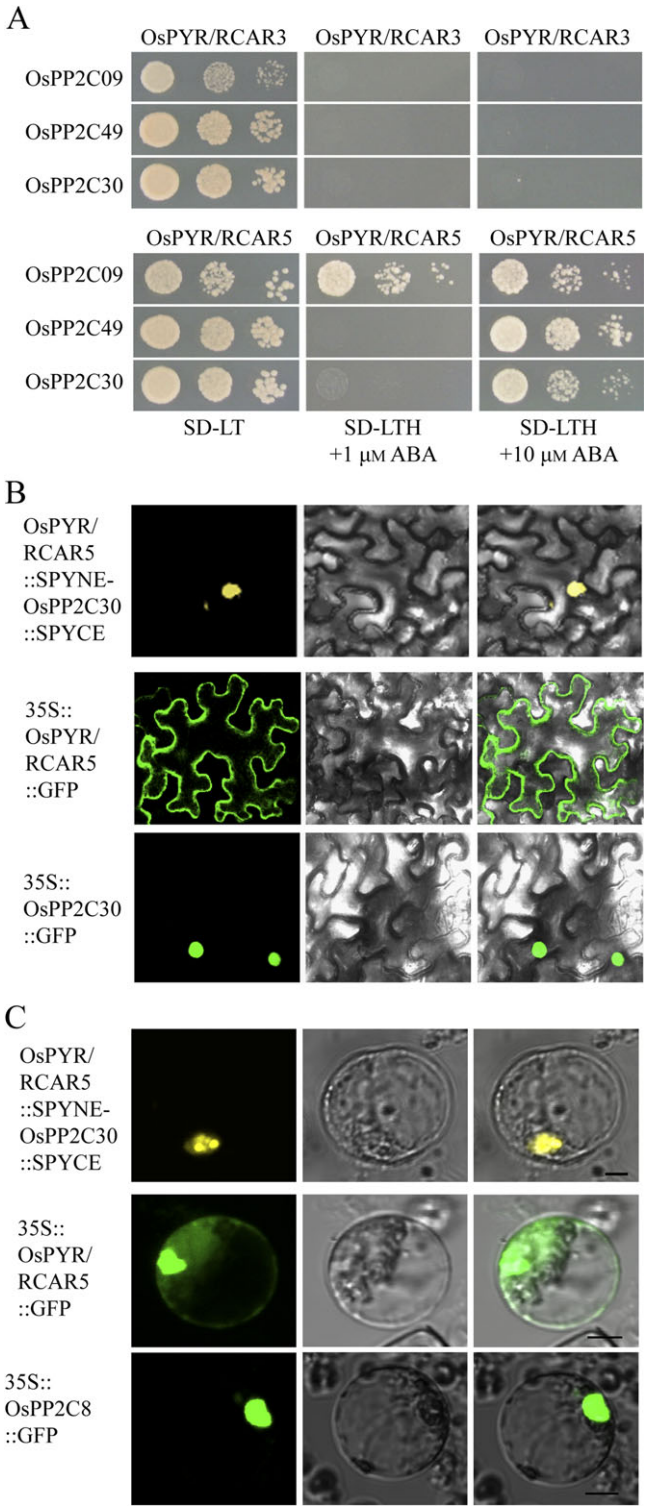

Fig. 4. OsPYL/RCAR5 and OsPP2C30 physically interact *in planta*. (A) Interaction of OsPYL/RCAR5 and OsPP2Cs in a yeast two-hybrid assay. Selection media were supplemented with different concentrations of ABA. Yeast two-hybrid assays with OsPYL/RCAR3 as bait and three OsPP2Cs as prey (upper panels), and with OsPYL/RCAR5 as bait and three OsPP2Cs as prey (lower panels) were conducted. (B) *In planta* interaction and subcellular localization analysis using agroinfiltrated *N. benthamiana* leaves. The interaction of OsPYL/RCAR5 and OsPP2C30 was detected by fluorescence in bimolecular fluorescence complementation (BiFC) analysis (upper panels). Subcellular localization analysis of OsPYL/RCAR5::GFP (middle panels) and OsPP2C30::GFP (lower panels) is shown. (C) *In planta* interaction and subcellular localization analysis using rice protoplasts. The interaction of OsPYL/RCAR5 and OsPP2C30 was detected by fluorescence using BiFC analysis (upper panels). Subcellular localization analysis of OsPYL/RCAR5::GFP (middle pannels) and OsPP2C30::GFP (lower panels) is shown in rice protoplasts. Bars=10 μm.

application of ABA caused no changes in the complex interaction or subcellular localization (data not shown). This result is similar to the subcellular localization of the FsPP2C1–PYL8/RCAR3 complex. The complex localization was also not affected in the absence or presence of ABA (Saavedra *et al.*, 2010). It is assumed that the agroinfiltrated tobacco leaves and rice protoplasts might have a sufficient physiological quantity of ABA to induce the interaction between OsPYL/RCAR5 and OsPP2C30 since agroinfiltration and making the protoplasts cause quite harsh wounding and stress to the cell.

These subcellular localization results were more or less identical in the two different systems of tobacco and rice. Consequently, it is possible that OsPP2C30 might determine the subcellular localization of the OsPYL/RCAR5–OsPP2C30 complex to the nucleus and that the OsPYL/RCAR5–OsPP2C30 complex functions in the nucleus in ABA signalling.

SAPK2 can induce ABA-dependent gene expression mediated by OREB1

SnRK2 subclass II and subclass III have been identified as signalling kinases involved in ABA signalling in *Arabidopsis* (Boudsocq *et al.*, 2004; Kobayashi *et al.*, 2004, 2005). However, only SnRK2 subclass III was known to be involved in ABA signalling. In order to determine whether SnRK2 subclass I or II plays a specific role in ABA-dependent transcription regulation, SAPKs were screened for ABRE-LUC reporter activation through OREB1 using transient gene expression in an *A. thaliana* protoplast (TEAMP) system. Although a higher order of mutant protoplasts that reduces gene redundancy is ideal for this assay, it was not possible to establish a relatively weak but clear transient activation assay using wild-type protoplasts. When SAPK2, SAPK4, OSRK1/SAPK6, and SAPK7 were individually transfected along with OREB1 and ABRE-LUC and expressed in *Arabidopsis* protoplasts, SAPK2, a member of SnRK2 subclass II, could induce more than three times as much luciferase gene expression as was observed with the other SAPKs, members of SnRK2 subclass I (Fig. 5A, see Supplementary Fig. S3 at *JXB* online). This induction was noted even in the absence of ABA, and was enhanced much more in the presence of ABA. The results demonstrate that SAPK2 may predominantly mediate ABA signalling, relative to the other SAPKs tested herein.

To verify the function of SAPK2 in ABA-dependent transcription, ABRE-LUC reporter activity was re-examined using a different combination of signalling components from those employed in the previous screening (Fig. 5A). Three different combinations of constructs [(i) reporter; (ii) reporter and OREB1; and (iii) reporter, OREB1, and SAPK2] were transfected into *Arabidopsis* protoplasts. Although the OREB1 and reporter co-transfection resulted in a slight induction of the reporter upon ABA supplementation, the addition of SAPK2 markedly increased the induction of the reporter gene, and ABA supplementation increased gene induction 2-fold compared with that observed in the absence of ABA (Fig. 5B). These results indicate that SAPK2, a SnRK2 subclass II member, could mediate the ABA signal transduction pathway via OREB1 and induces ABA-dependent ABRE promoter activity in rice.

SAPK2 interacts physically with OsPP2C30 and OREB1

In *Arabidopsis*, interaction and phospho/dephosphorylation between SnRK2s and clade A PP2Cs is one of the principal regulatory mechanisms for ABA signalling. In order to determine whether SAPK2 interacts with OsPP2C30, BiFC assays in tobacco epidermis and rice protoplasts were carried out. Fluorescence was detected for the SAPK2 and OsPP2C30 combination of BiFC, and the complex of SAPK2 and OsPP2C30 was clearly detected within the nuclei of both tobacco epidermis cells and rice protoplasts, consistent with OsPYL/RCAR5 and OsPP2C30, whereas SAPK2::GFP is localized within both the cytosol and the nucleus (Fig. 6). In order to check whether ABA can change the localization of SAPK2::GFP, 1 μM ABA was added to the transfected protoplasts. The application of ABA caused no changes in its subcellular localization (data not shown). These localization results demonstrate that OsPP2C30 might determine the subcellular localization of the SAPK2–OsPP2C30 and OsPYL/RCAR5–OsPP2C30 complexes to the nucleus, and these complexes function in the nucleus in ABA-dependent gene expression.

It was determined that SAPK2 might be involved in ABA-dependent gene expression through OREB1 using the TEAMP system. Thus, an attempt was made to determine whether SAPK2 interacts with OREB1. It was also determined that OREB1 is an interacting partner of SAPK2 using the BiFC assay (Fig. 6A). As for the other proteins, this complex is localized within the nuclei of tobacco epidermis cells and rice protoplasts, and OREB1::GFP is localized in the nucleus (Fig. 6A, B).

Reconstitution of the rice ABA signal transduction pathway consisting of OsPYL/RCAR5, PP2C30, SAPK2, and OREB1

The data above suggest the existence of an ABA signal transduction pathway consisting of OsPYL/RCAR5, OsPP2C30, SAPK2, and OREB1. To verify that these are a unit of the ABA signal transduction pathway in rice, an attempt was made to reconstitute the signalling cascade using the TEAMP system. When OsPP2C30 was co-transfected with SAPK2, OREB1, and ABRE-LUC, reporter activity was abolished in both the absence and presence of ABA (Fig. 7). This suggests that OsPP2C30 plays a negative role in ABA signalling. However, the induction of the reporter was restored by the additional expression of OsPYL/RCAR5 in the presence of ABA, but not in the absence of ABA. This suggests that OsPYL/RCAR5 is a positive regulator in ABA signalling. The results imply that the induction of the ABA-dependent reporter gene by OREB1 occurs in the context of ABA signalling via OsPYL/RCAR5, OsPP2C30, SAPK2, and OREB1. This is the first report to elucidate the minimal unit for the regulation of ABA-dependent gene expression in the context of rice ABA signalling.

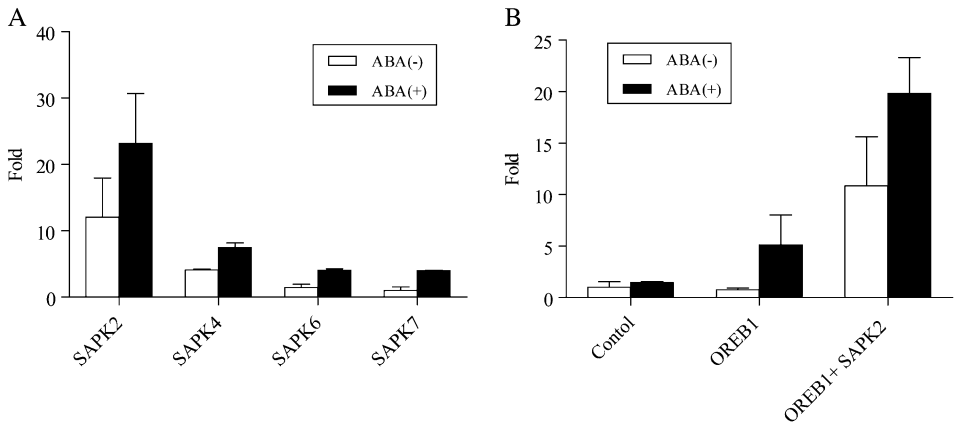

Fig. 5. SAPK2 induces ABA-dependent gene expression through OREB1. (A) SAPK2, SAPK4, SAPK6, and SAPK7 were screened along with OREB1 for ABRE-LUC induction in the absence and presence of ABA. (B) SAPK2 function in ABRE-LUC expression was further tested with and without OREB1 in the absence and presence of ABA. An empty vector and UBQ10-rLUC served as an effector and as a transfection control, respectively. Experiments were repeated twice with duplicated samples with similar results. Results are expressed as means ±SE.

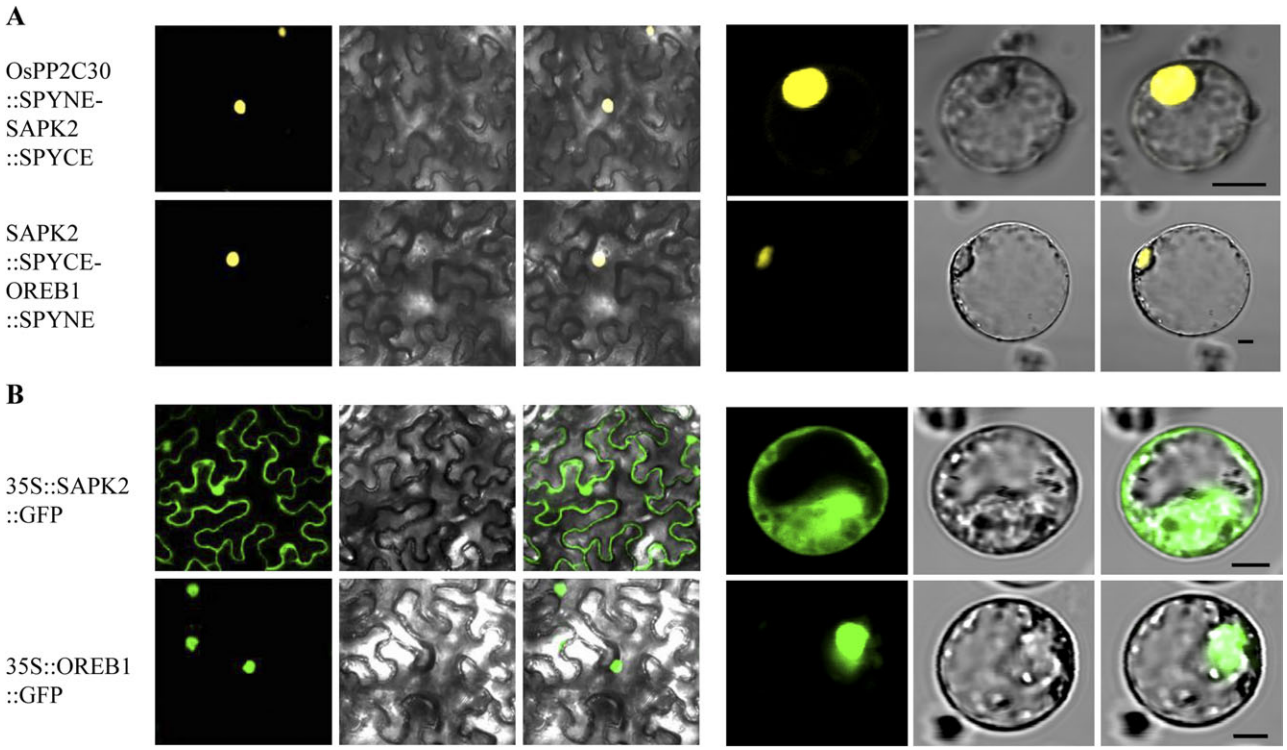

Fig. 6. SAPK2 interacts with OsPP2C30 and OREB1 *in planta*. (A) *In planta* interaction analysis via bimolecular fluorescence complementation using *N. benthamiana* leaves (left) and rice protoplasts (right). The interactions of OsPP2C30 and SAPK2 (upper images) and of OREB1 and SAPK2 (lower images) are presented. (B) Subcellular localization of SAPK2::GFP (upper images) and OREB1::GFP (lower images) in the leaf epidermis of tobacco (left) or rice protoplasts (right). Bar=10 μm.

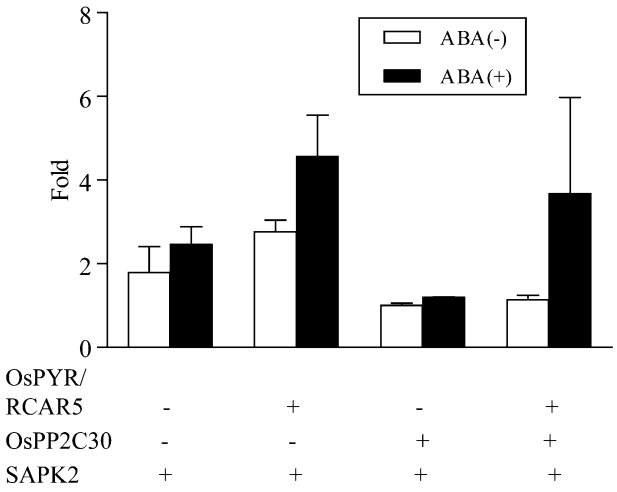

Fig. 7. Reconstitution of rice ABA signalling components in *Arabidopsis* protoplasts. ABRE-LUC induction via OsREB1 was examined in a combination of SAPK2, OsPYL/RCAR5, and OsPP2C30 in the presence and absence of ABA. An empty vector and UBQ10-rLUC served as effector and transfection controls, respectively. Experiments were repeated twice with duplicated samples with similar results. Results are expressed as means ±SE.

Discussion

Recently, the cellular ABA receptors, PYR/RCARs, were discovered in *Arabidopsis*. Thus, it has become an important issue to determine the function and molecular mechanisms of the ABA receptors in monocots. In this study, these questions were addressed using a rice system. Rice PYL/RCAR orthologues were identified based on amino acid sequence similarity, and it was demonstrated that OsPYL/RCAR5 might be a functional ABA receptor, with mechanisms similar to *Arabidopsis* PYL/RCARs in terms of ABA-dependent interaction with PP2Cs and the ABA-hypersensitive phenotype of transgenic plants that overexpress the gene. A minimal unit of the ABA signalling pathway in rice was also demonstrated by identifying the hierarchy and subcellular localization of interacting signalling components and reconstituting them in *Arabidopsis* protoplasts.

The signalling components for ABA-dependent transcriptional regulation are highly conserved in rice and Arabidopsis

The discovery of the *PYL/RCAR* gene family as ABA receptors provided a complete context beginning from ABA sensing, through signal transduction, and to the resultant gene expression (Ma *et al.*, 2009; Park *et al.*, 2009; Santiago *et al.*, 2009*a*). After the discovery of *Arabidopsis* PYL/RCAR, orthologues of these genes were identified in genomes ranging from mosses to crops. Other ABA signalling components such as PP2Cs and SnRK2s are also conserved from mosses to flowering plants, although the green alga *Chlamydomonas reinhardtii* does not have them. This suggests that ABA signalling components may have

arisen as plants evolved from aquatic organisms to terrestrial organisms (Klingler *et al.*, 2010; Umezawa *et al.*, 2010). There appear to be 13 orthologues of PYL/RCAR, 10 PP2Cs, 10 SnRK2s, and 11 ABF-like bZIP transcription factors in rice (see Supplementary Figs S1, S2, and S4 at *JXB* online). The number of the members in each of the subgroups is also quite similar in *Arabidopsis* and rice. These ABA signalling components are conserved in terms of functional mechanisms, as well as amino acid sequences, among plant species. Beechnut FsPP2C1 was shown to interact with *Arabidopsis* PYL8/RCAR3, and *Arabidopsis* plants overexpressing the gene showed an ABA-insensitive phenotype, thereby suggesting that FsPP2C1 can function as a negative regulator of ABA signalling in *Arabidopsis*. These results demonstrate that FsPP2C1 can function through interaction with AtPYL/RCAR, even in different species (Gonzalez-Garcia *et al.*, 2003; Saavedra *et al.*, 2010). FsPP2C1 is placed in the same subgroup with OsPP2C30 and AtAHG3, and OsPYL/RCAR5 is a member of the same subgroup as PYL8/RCAR3. The phenotype of *Arabidopsis* seeds overexpressing PYL8/RCAR3 was quite similar to that observed in the *OsPYL/RCAR5*-overexpressing transgenic rice lines in terms of seed germination and early seedling growth. Furthermore, the nuclear localization of the OsPYL/RCAR5–OsPP2C30 complex is the same as that of the beechnut FsPP2C1–PYL8/RCAR3 complex, and is particularly striking compared with the ABI1–RCAR1 complex localized within the cytosol and nucleus (Ma *et al.*, 2009; Saavedra *et al.*, 2010). Taken together, OsPYL/RCAR5 and PYL8/RCAR3 may perform the same biological functions in seed germination and early seedling growth along with FsPP2C1, OsPP2C30, and AHG3 in *Arabidopsis*, beechnut, and rice, respectively. It was also demonstrated that the PYL/RCAR, PP2CA, SnRK2, and bZIP transcription factors were the minimal components for the regulation of the ABA-dependent gene expression via the reconstitution of the components in the *Arabidopsis* protoplast (Fujii *et al.*, 2009). In this study, OsPYL/RCAR5, OsPP2C30, SAPK2, and OREB1 were also reconstituted in *Arabidopsis* protoplasts, and those components are a minimal functional unit for ABA sensing, ABA signal transduction, and the activation of ABRE-dependent transcription in rice. Thus, it appears likely that the ABA signal transduction pathway mediated by PYL/RCAR and PP2C is highly conserved in *Arabidopsis* and rice in terms of biological function, sequence, and functional mechanisms.

SnRK2 subclass II members could regulate ABA-responsive gene expression mediated by OREB1 in rice

SnRK2s are known as the principal positive regulators of ABA and osmotic stress in *Arabidopsis* and rice. This kinase family consists of 10 genes classified into three subclasses in rice and *Arabidopsis* (Kobayashi *et al.*, 2004). Members of SnRK2 subclasses II and III showed kinase activity in *Arabidopsis* under ABA and osmotic stress conditions. In particular, subclass III SnRK2s, including OST1/SnRK2.6, are known as the principal regulators of ABA-dependent signal transduction (Boudsocq *et al.*, 2004). In contrast to *Arabidopsis*, only SnRK2 subclass III members, SAPK 8, 9, and 10, have been shown to be activated by ABA in rice, and subclass II and I members are activated only by osmotic and salt stress (Kobayashi *et al.*, 2004). However, several reports have indicated that SnRK2 subclass I or II members could be involved in ABA-dependent gene expression through OREB1. It was reported that SAPK4 interacts with OREB1 in yeast and plants, and that OSRK1/SAPK6 can phosphorylate OREB1 under *in vitro* conditions (Chae *et al.*, 2007; Ding *et al.*, 2009; Hong *et al.*, 2011). Another report revealed that wheat PKABA1, a member of SnRK2 subclass II, interacts with and phosphorylates TaABF, a homologue of OREB1 (Johnson *et al.*, 2002; Kobayashi *et al.*, 2005).

In the present study, SAPK2, a member of SnRK2 subclass II, was shown to be able to activate OREB1 to induce the ABA-dependent gene expression of the reporter luciferase fused to ABRE. This activation is much stronger than that of the SnRK2 subclass I members. Co-transfection of *OsPP2C30* with *SAPK2* diminished reporter gene expression, and the addition of *OsPYL/RCAR5* induced its recovery, depending on ABA. These results indicate that members of SnRK2 subclass II, as well as members of SnRK2 subclass III, are involved in the regulation of ABA-dependent gene expression via the ABA signal transduction pathway mediated by OsPYL/RCARs and PP2Cs in rice.

Complexes of ABA signalling components are functional in the nucleus

OsPP2C30 was localized to the nucleus and its complexes were also localized to the nucleus, even if the interacting partners were not exclusively localized within the nucleus. This indicates that the complexes consisting of OsPP2C30 and its interacting partners, SAPK2 and OsPYL/RCAR5, are functional in the nucleus, where they can function in regulating ABA-dependent gene expression (Fig. 8). OsPP2C30 and several members of the clade A OsPP2C family have putative nuclear localization sequences (NLSs) different from those of the clade A PP2C of *Arabidopsis*. However, other components, including OsPYL/RCAR5 and SAPK2, do not have NLSs and are localized in both the cytosol and the nucleus. Thus, it appears likely that OsPP2C30 determines the nuclear localization of the complex. This result indicates that OsPYL/RCAR5 may function as a nuclear ABA receptor in the regulation of gene expression mediated by OsPP2C30 (Cutler *et al.*, 2010). In *Arabidopsis*, HAB1, a member of the clade A PP2C family, regulates gene expression via chromatin remodelling through direct interaction with SWI1, a homologue of the yeast SWI3 subunit of SWI/SNF chromatin-remodelling complexes in the nuclei (Saez *et al.*, 2008). Although only a few PP2Cs, including FsPP2C1 and the mitogen-activated protein kinase (MAPK) phosphatase PP2C5, have been reported to perform roles in the nucleus in plants, several phosphatases are known to be involved in the regulation of nuclear processes including gene

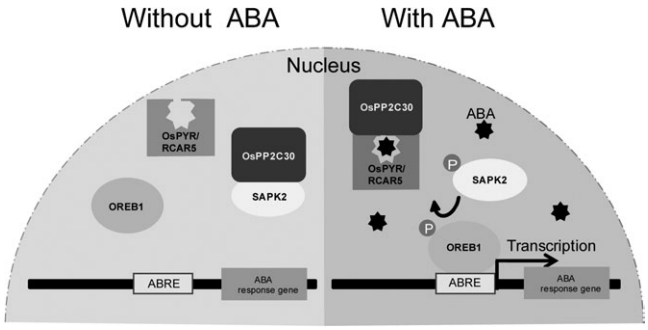

Fig. 8. A proposed working model for ABA-dependent gene regulation mediated by OsPYL/RCAR5 in the rice nucleus. ABA binds to OsPYL/RCAR5 and induces complex formation with PP2C30 in the nucleus. Phosphorylated SAPK2 activates OREB1 and then the ABA-responsive genes are expressed.

transcription, RNA processing, and DNA damage processing in other eukaryote systems (Moorhead *et al.*, 2007; Brock *et al.*, 2010; Saavedra *et al.*, 2010). Thus, it is supposed that OsPP2C30 might function in ABA signalling via other mechanisms, including chromatin remodelling and ABRE-dependent gene expression regulation. This study demonstrated that the ABA signal transduction pathway involving the clade A PP2Cs and the ABA receptors is highly conserved in terms of components and functional mechanisms in monocot and dicot plants. This study will provide useful information for stress-tolerant crop development via the manipulation of the ABA signal transduction pathway.

Supplementary data

Supplementary data are available at *JXB* online.

Figure S1. OsPYR/RCARs have high similarity to *Arabidopsis* PYR/RCARs.

Figure S2. Phylogenic tree and expression levels of clade A OsPP2Cs.

Figure S3. Transient expression of the ABA signalling components.

Figure S4. Phylogenic tree of ABF-like bZIP transcription factors in *Arabidopsis* and rice.

Table S1. Primer sequences used for real-time PCR and RT-PCR.

Acknowledgements

We thank G.-H. An for providing the pGA2897 vector and Jorg Kudla for providing the BiFC vectors. This work was supported by the Agenda Project (PJ006647) National Academy of Agricultural Science, Rural Development Administration to B-GK, and HH was supported by the 2011 Postdoctoral Fellowship Program of NAAS, RDA.

References

Adie BA, Perez-Perez J, Perez-Perez MM, Godoy M, Sanchez-Serrano JJ, Schmelz EA, Solano R. 2007. ABA is an essential signal for plant resistance to pathogens affecting JA biosynthesis and the activation of defenses in Arabidopsis. *The Plant Cell* **19,** 1665–1681.

Boudsocq M, Barbier-Brygoo H, Lauriere C. 2004. Identification of nine sucrose nonfermenting 1-related protein kinases 2 activated by hyperosmotic and saline stresses in Arabidopsis thaliana. *Journal of Biological Chemistry* **279,** 41758–41766.

Brock AK, Willmann R, Kolb D, Grefen L, Lajunen HM, Bethke G, Lee J, Nurnberger T, Gust AA. 2010. The Arabidopsis mitogen-activated protein kinase phosphatase PP2C5 affects seed germination, stomatal aperture, and abscisic acid-inducible gene expression. *Plant Physiology* **153,** 1098–1111.

Chae MJ, Lee JS, Nam MH, Cho K, Hong JY, Yi SA, Suh SC, Yoon IS. 2007. A rice dehydration-inducible SNF1-related protein kinase 2 phosphorylates an abscisic acid responsive element-binding factor and associates with ABA signaling. *Plant Molecular Biology* **63,** 151–169.

Chen S, Tao L, Zeng L, Vega-Sanchez ME, Umemura K, Wang GL. 2006. A highly efficient transient protoplast system for analyzing defence gene expression and protein–protein interactions in rice. *Molecual Plant Pathology* **7,** 417–427.

Cutler SR, Rodriguez PL, Finkelstein RR, Abrams SR. 2010. Abscisic acid: emergence of a core signaling network. *Annual Review of Plant Biology* **61,** 651–679.

Ding X, Richter T, Chen M, *et al*. 2009. A rice kinase–protein interaction map. *Plant Physiology* **149,** 1478–1492.

Fujii H, Chinnusamy V, Rodrigues A, Rubio S, Antoni R, Park SY, Cutler SR, Sheen J, Rodriguez PL, Zhu JK. 2009. *In vitro* reconstitution of an abscisic acid signalling pathway. *Nature* **462,** 660–664.

Fukao T, Yeung E, Bailey-Serres J. 2011. The submergence tolerance regulator SUB1A mediates crosstalk between submergence and drought tolerance in rice. *The Plant Cell* **23,** 412–427.

Gonzalez-Garcia MP, Rodriguez D, Nicolas C, Rodriguez PL, Nicolas G, Lorenzo O. 2003. Negative regulation of abscisic acid signaling by the Fagus sylvatica FsPP2C1 plays a role in seed dormancy regulation and promotion of seed germination. *Plant Physiology* **133,** 135–144.

Hong JY, Chae MJ, Lee IS, Lee YN, Nam MH, Kim DY, Byun MO, Yoon IS. 2011. Phosphorylation-mediated regulation of a rice ABA responsive element binding factor. *Phytochemistry* **72,** 27–36.

Johnson RR, Wagner RL, Verhey SD, Walker-Simmons MK. 2002. The abscisic acid-responsive kinase PKABA1 interacts with a seed-specific abscisic acid response element-binding factor, TaABF, and phosphorylates TaABF peptide sequences. *Plant Physiology* **130,** 837–846.

Kagaya Y, Hobo T, Murata M, Ban A, Hattori T. 2002. Abscisic acid-induced transcription is mediated by phosphorylation of an abscisic acid response element binding factor, TRAB1. *The Plant Cell* **14,** 3177–3189.

Klingler JP, Batelli G, Zhu JK. 2010. ABA receptors: the START of a new paradigm in phytohormone signalling. *Journal of Experimental Botany* **61,** 3199–3210.

Kobayashi Y, Murata M, Minami H, Yamamoto S, Kagaya Y, Hobo T, Yamamoto A, Hattori T. 2005. Abscisic acid-activated SNRK2 protein kinases function in the gene-regulation pathway of ABA signal transduction by phosphorylating ABA response element-binding factors. *The Plant Journal* **44,** 939–949.

Kobayashi Y, Yamamoto S, Minami H, Kagaya Y, Hattori T. 2004. Differential activation of the rice sucrose nonfermenting1-related protein kinase2 family by hyperosmotic stress and abscisic acid. *The Plant Cell* **16,** 1163–1177.

Kuhn JM, Boisson-Dernier A, Dizon MB, Maktabi MH, Schroeder JI. 2006. The protein phosphatase AtPP2CA negatively regulates abscisic acid signal transduction in Arabidopsis, and effects of abh1 on AtPP2CA mRNA. *Plant Physiology* **140,** 127–139.

Ma Y, Szostkiewicz I, Korte A, Moes D, Yang Y, Christmann A, Grill E. 2009. Regulators of PP2C phosphatase activity function as abscisic acid sensors. *Science* **324,** 1064–1068.

McCourt P, Creelman R. 2008. The ABA receptors—we report you decide. *Current Opinion in Plant Biology* **11,** 474–478.

Melcher K, Ng LM, Zhou XE, *et al*. 2009. A gate–latch–lock mechanism for hormone signalling by abscisic acid receptors. *Nature* **462,** 602–608.

Melcher K, Xu Y, Ng LM, *et al*. 2010. Identification and mechanism of ABA receptor antagonism. *Nature Structural and Molecular Biology* **17,** 1102–1108.

Miyazono K, Miyakawa T, Sawano Y, *et al*. 2009. Structural basis of abscisic acid signalling. *Nature* **462,** 609–614.

Moorhead GB, Trinkle-Mulcahy L, Ulke-Lemee A. 2007. Emerging roles of nuclear protein phosphatases. *Nature Reviews. Molecular Cell Biology* **8,** 234–244.

Nakashima K, Ito Y, Yamaguchi-Shinozaki K. 2009. Transcriptional regulatory networks in response to abiotic stresses in Arabidopsis and grasses. *Plant Physiology* **149,** 88–95.

Nishimura N, Hitomi K, Arvai AS, Rambo RP, Hitomi C, Cutler SR, Schroeder JI, Getzoff ED. 2009. Structural mechanism of abscisic acid binding and signaling by dimeric PYR1. *Science* **326,** 1373–1379.

Pandey S, Nelson DC, Assmann SM. 2009. Two novel GPCR-type G proteins are abscisic acid receptors in Arabidopsis. *Cell* **136,** 136–148.

Park SY, Fung P, Nishimura N, *et al*. 2009. Abscisic acid inhibits type 2C protein phosphatases via the PYR/PYL family of START proteins. *Science* **324,** 1068–1071.

Peterson FC, Burgie ES, Park SY, Jensen DR, Weiner JJ, Bingman CA, Chang CE, Cutler SR, Phillips Jr GN, Volkman BF. 2010. Structural basis for selective activation of ABA receptors. *Nature Structural and Molecular Biology* **17,** 1109–1113.

Rodriguez-Gacio Mdel C, Matilla-Vazquez MA, Matilla AJ. 2009. Seed dormancy and ABA signaling: the breakthrough goes on. *Plant Signaling and Behavior* **4,** 1035–1049.

Saavedra X, Modrego A, Rodriguez D, Gonzalez-Garcia MP, Sanz L, Nicolas G, Lorenzo O. 2010. The nuclear interactor PYL8/RCAR3 of Fagus sylvatica FsPP2C1 is a positive regulator of abscisic acid signaling in seeds and stress. *Plant Physiology* **152,** 133–150.

Saez A, Rodrigues A, Santiago J, Rubio S, Rodriguez PL. 2008. HAB1–SWI3B interaction reveals a link between abscisic acid signaling and putative SWI/SNF chromatin-remodeling complexes in Arabidopsis. *The Plant Cell* **20,** 2972–2988.

Santiago J, Dupeux F, Round A, Antoni R, Park SY, Jamin M, Cutler SR, Rodriguez PL, Marquez JA. 2009*a*. The abscisic acid receptor PYR1 in complex with abscisic acid. *Nature* **462,** 665–668.

Santiago J, Rodrigues A, Saez A, Rubio S, Antoni R, Dupeux F, Park SY, Marquez JA, Cutler SR, Rodriguez PL. 2009*b*. Modulation of drought resistance by the abscisic acid receptor PYL5 through inhibition of clade A PP2Cs. *The Plant Journal* **60,** 575–588.

Sirichandra C, Davanture M, Turk BE, Zivy M, Valot B, Leung J, Merlot S. 2010. The Arabidopsis ABA-activated kinase OST1 phosphorylates the bZIP transcription factor ABF3 and creates a 14-3-3 binding site involved in its turnover. *PLoS One* **5,** e13935.

Toki S, Hara N, Ono K, Onodera H, Tagiri A, Oka S, Tanaka H. 2006. Early infection of scutellum tissue with Agrobacterium allows high-speed transformation of rice. *The Plant Journal* **47,** 969–976.

Umezawa T, Nakashima K, Miyakawa T, Kuromori T, Tanokura M, Shinozaki K, Yamaguchi-Shinozaki K. 2010. Molecular basis of the core regulatory network in ABA responses: sensing, signaling and transport. *Plant and Cell Physiology* **51,** 1821–1839.

Umezawa T, Sugiyama N, Mizoguchi M, Hayashi S, Myouga F, Yamaguchi-Shinozaki K, Ishihama Y, Hirayama T, Shinozaki K. 2009. Type 2C protein phosphatases directly regulate abscisic acid-activated protein kinases in Arabidopsis. *Proceedings of the National Academy of Sciences, USA* **106,** 17588–17593.

Waadt R, Schmidt LK, Lohse M, Hashimoto K, Bock R, Kudla J. 2008. Multicolor bimolecular fluorescence complementation reveals simultaneous formation of alternative CBL/CIPK complexes in planta. *The Plant Journal* **56,** 505–516.

Xue T, Wang D, Zhang S, Ehlting J, Ni F, Jakab S, Zheng C, Zhong Y. 2008. Genome-wide and expression analysis of protein phosphatase 2C in rice and Arabidopsis. *BMC Genomics* **9,** 550.

Yoo SD, Cho YH, Sheen J. 2007. Arabidopsis mesophyll protoplasts: a versatile cell system for transient gene expression analysis. *Nature Protocols* **2,** 1565–1572.

Yoshida T, Nishimura N, Kitahata N, Kuromori T, Ito T, Asami T, Shinozaki K, Hirayama T. 2006. ABA-hypersensitive germination3 encodes a protein phosphatase 2C (AtPP2CA) that strongly regulates abscisic acid signaling during germination among Arabidopsis protein phosphatase 2Cs. *Plant Physiology* **140,** 115–126.