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Transgenic expression of rice *MYB102* (*OsMYB102*) delays leaf senescence and decreases abiotic stress tolerance in *Arabidopsis thaliana*

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MYB-type transcription factors (TFs) play important roles in plant growth and development, and in the rapid responses to unfavorable environmental conditions. We recently reported the isolation and characterization of a rice (*Oryza sativa*) MYB TF, OsMYB102, which is involved in the regulation of leaf senescence by downregulating abscisic acid (ABA) biosynthesis and the downstream signaling response. Based on the similarities of their sequences and expression patterns, OsMYB102 appears to be a homolog of the *Arabidopsis thaliana* AtMYB44 TF. Since AtMYB44 is a key regulator of leaf senescence and abiotic stress responses, it is important to examine whether AtMYB44 homologs in other plants also act similarly. Here, we generated transgenic Arabidopsis plants expressing *OsMYB102* (*OsMYB102*-OX). The *OsMYB102*-OX plants showed a delayed senescence phenotype during dark incubation and were more susceptible to salt and drought stresses, considerably similar to Arabidopsis plants overexpressing *AtMYB44*. Real-time quantitative PCR (RT-qPCR) revealed that, in addition to known senescence-associated genes, genes encoding the ABA catabolic enzymes AtCYP707A3 and AtCYP707A4 were also significantly upregulated in *OsMYB102*-OX, leading to a significant decrease in ABA accumulation. Furthermore, protoplast transient expression and chromatin immunoprecipitation assays revealed that OsMYB102 directly activated *AtCYP707A3* expression. Based on our findings, it is probable that the regulatory functions of AtMYB44 homologs in plants are highly conserved and they have vital roles in leaf senescence and the abiotic stress responses. [BMB Reports 2019; 52(11): 653-658]

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INTRODUCTION

Transcription factors (TFs) are involved in the regulation of gene expression and generally consist of two main domains, the DNA-binding and activation/repression domains (1). In plants, the MYB superfamily is one of the largest TF families, with approximately 198 members in *Arabidopsis thaliana* and 183 members in rice (*Oryza sativa*) (2). Plant MYB TFs regulate multiple biological processes, such as biotic/abiotic stress response, cell cycle, hormone signaling, and secondary metabolism (3-6). Some transcriptomic analyses have also indicated the importance of the MYB TFs during the onset of leaf senescence (7, 8). In Arabidopsis, AtMYBH was identified as a up-regulator of leaf senescence, with the *atmybh* mutants retaining leaf green color much longer and the *AtMYBH*-overexpressing lines exhibited an early leaf senescing phenotype during natural and dark-induced leaf senescence (9). By contrast, AtMYB44 is a down-regulator of leaf senescence, since Arabidopsis transgenics overexpressing *AtMYB44* delayed leaf senescence and the *atmyb44* mutants senesced precociously (10). In addition, AtMYB44 directly represses the transcription of senescence-associated genes (SAGs), including *WRKY70* and *ETHYLENE INSENSITIVE 2* (*EIN2*) (11, 12).

Although little is known about the senescence-associated MYB TFs in crop plants, we previously revealed that a rice MYB TF, OsMYB102, is involved the regulation of leaf senescence (13). A T-DNA-mediated activation-tagging line of *OsMYB102* (*osmyb102-D*) and *OsMYB102*-overexpressing transgenic plants showed a strong delayed senescence phenotype during both natural and artificially induced leaf senescence (13). In addition, OsMYB102 was found to directly activate the expression of *OsCYP707A6*, encoding an ABA catabolic enzyme, and to indirectly repress the transcription of *OsNAP* and *OsABF4*, which encode TFs associated with the response to ABA signaling (13). OsMYB102 therefore appears to delay leaf yellowing by inhibiting both ABA concentration and ABA signaling response. Through the genome-wide comparison of MYB TFs between Arabidopsis and

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rice, it revealed that OsMYB102 is a high candidate of AtMYB44 orthologue (14), and their R2R3 domains have 81% identity. As described above. AtMYB44 acts as a key regulator of leaf senescence and abiotic stress responses in Arabidopsis, but it is still largely unknown whether AtMYB44 homolog of other plant species act similarly.

In this study, we generated transgenic Arabidopsis plants expressing *OsMYB102* (hereafter *OsMYB102*-OX) to examine whether OsMYB102 plays a similar role in the regulation of leaf senescence in both rice and Arabidopsis. We found that the *OsMYB102*-OX plants displayed a delayed leaf senescence phenotype during dark-induced senescence (DIS), and were hypersensitive to salinity and drought stresses. Using a transcriptomic analysis, we found that a number of genes related to ABA metabolism and signaling were differentially expressed in *OsMYB102*-OX. Furthermore, we used chromatin immunoprecipitation (ChIP) and protoplast transactivation assays to reveal that OsMYB102 directly binds to the promoter of *AtCYP707A3* to activate its transcription. We discuss the similarities and differences of the roles of OsMYB102 in rice and AtMYB44 in Arabidopsis.

RESULTS

Transgenic expression of *OsMYB102* in Arabidopsis inhibits shoot growth

OsMYB102 is an R2R3-type MYB TF in rice and the closest homolog of AtMYB44 in Arabidopsis. The expression patterns of *OsMYB102* and *AtMYB44* are also similar: Their transcripts are abundant both in leaves and roots (10) (Supplementary Fig. S1), and are also strongly induced by ABA treatment (13, 14), during DIS (Supplementary Fig. S2) (13), and under high salinity and drought stresses (Supplementary Fig. S3) (14). Thus, it is possible that OsMYB102 acts similar to AtMYB44 in Arabidopsis.

To examine the conserved function of OsMYB102 in Arabidopsis, we generated transgenic Arabidopsis plants that constitutively expressed *OsMYB102* from a *35S*:*OsMYB102* construct. We used reverse transcription-quantitative real-time PCR (RT-qPCR) analyses to identify three independent *OsMYB102*-OX lines (Fig. 1A), and showed that, at the vegetative stage, these three *OsMYB102*-OX lines were all significantly smaller than the wild type (WT) and had shorter petioles (Fig. 1B, C). In addition, the fresh weights of the *OsMYB102*-OX plants were lower than the WT throughout their development (Fig. 1D), indicating that the constitutive expression of *OsMYB102* has a negative effect on shoot growth in Arabidopsis.

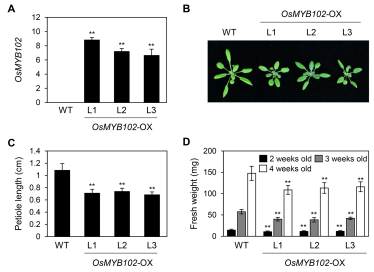

Fig. 1. The transgenic expression of *OsMYB102* inhibits plant growth in Arabidopsis. Plants were grown under long-day (LD) conditions (16 h light/8 h light). (A) The relative transcript levels of *OsMYB102* in three-week-old Col-0 (WT) plants and three independent *OsMYB102*-OX lines were determined using RT-qPCR and normalized to the transcript levels of *AtGAPDH*. The mean and SD values were obtained from more than three biological samples. (B, C) Visible phenotypes (B) and petiole lengths (C) of the WT and three independent *OsMYB102*-OX lines. (D) Fresh weights of two-, three-, and four-week-old WT and three independent *OsMYB102*-OX plants. Asterisks indicate a significant difference between WT and *OsMYB102*-OX plants (Student's *t*-test, *P < 0.05, **P < 0.01). These experiments were repeated three times with similar results.

OsMYB102-OX plants have delayed leaf senescence

OsMYB102 was previously shown to delay leaf senescence by inhibiting ABA accumulation and signaling in rice (13); therefore, we examined the phenotype of the *OsMYB102*-OX plants during DIS. Four-week-old plants were transferred into complete darkness to induce artificial senescence. After 6 d of dark incubation (6 DDI), the WT leaves had turned yellow while the *OsMYB102*-OX leaves retained much of their greenness (Fig. 2A). Similar stay-green phenotypes were observed using rosette leaves detached from four-week-old *OsMYB102*-OX plants at 4 DDI (Fig. 2B). Consistent with the visible phenotype, the

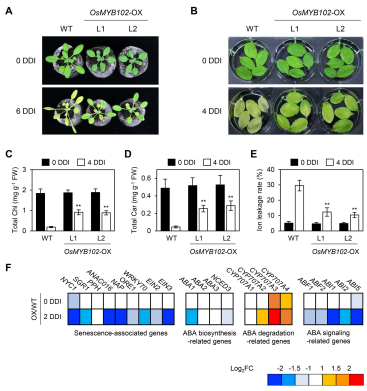

Fig. 2. The transgenic expression of *OsMYB102* in Arabidopsis delays leaf senescence. (A) Four-week-old Col-0 (WT) and two independent *OsMYB102*-OX lines were transferred into complete darkness, and their visible phenotypes were observed at 6 d of dark incubation (DDI). (B-E) Changes in the leaf color (B), total chlorophyll (Chl) content (C), total carotenoid (Car) content (D), and membrane ion leakage rate (E) in the detached rosette leaves of three-week-old WT and *OsMYB102*-OX plants at 0 and 4 DDI. The mean and SD values were obtained from more than five biological samples. Asterisks indicate a significant difference between the WT and *OsMYB102*-OX (Student's *t*-test, *P < 0.05, **P < 0.01). (F) The relative expression levels (*OsMYB102*-OX/WT) of the senescence-associated genes (SAGs) and ABA metabolism- and signaling-related genes at 0 and 2 DDI in *OsMYB102*-OX and the WT. These experiments were repeated twice with similar results.

detached *OsMYB102*-OX leaves retained significantly more of their total chlorophyll (Chl) and total carotenoids (Car), while showing a lower ion leakage rate than the WT leaves (Fig. 2C-E). These findings indicate that *OsMYB102* can delay leaf senescence in Arabidopsis, similar to its function in rice.

To understand how OsMYB102 delays leaf senescence in Arabidopsis, we first examined the expression patterns of the SAGs in the *OsMYB102*-OX plants during DIS (Fig. 2F). In addition, we checked the expression patterns of genes related to ABA biosynthesis, degradation, and signaling , since OsMYB102 mainly affected the expression of the ABA-associated genes in rice (13). At 2 DDI, several SAGs, such as *NON-YELLOW COLORING 1* (*AtNYC1*), encoding a Chl catabolic enzyme (15); *AtNAP* and *AtANAC016*, encoding senescence-associated NAC TFs (16, 17); and *EIN3*, encoding a key TF in ethylene-induced leaf senescence (18), were all significantly downregulated in the *OsMYB102*-OX leaves. The ABA signaling-related genes, including *ABA INSENSITIVE 1* (*AtABI1*) and *AtABI5*, were also downregulated, while the ABA degradation-related genes (*AtCYP707A3* and *AtCYP707A4*) were significantly upregulated in the *OsMYB102*-OX leaves. Remarkably, the expression levels of *AtCYP707A3* and *AtCYP707A4* in *OsMYB102*-OX were higher than they were in the WT, even at 0 DDI. Taken together, these results suggest that the OsMYB102 TF functions to delay leaf senescence in Arabidopsis by modulating the expression of a number of SAGs and ABA degradation- and signaling-related genes.

OsMYB102-OX plants have decreased abiotic stress tolerance

ABA is a phytohormone with a vital role in the regulation of abiotic stress responses (19). Our finding that a number of genes involved in ABA metabolism and signaling were differentially expressed in the *OsMYB102*-OX leaves during DIS led us to examine the phenotype of the *OsMYB102*-OX plants under abiotic stress conditions, such as high salinity and drought stresses. When placed in Murashige and Skoog (MS) medium containing 150 mM NaCl, the detached rosette leaves from the *OsMYB102*-OX plants became necrotic much faster than the WT leaves (Fig. 3A), resulting in a lower Chl content (Fig. 3B). A similar phenotype was also observed in young seedlings subjected to salt stress; when 5-d-old seedlings grown on MS agar plates were transferred to plates containing 100 mM NaCl, the *OsMYB102*-OX seedlings were hypersensitive to the high salinity stress (Fig. 3C). Consistent with the visible phenotype, the *OsMYB102*-OX seedlings had a lower Chl content (Fig. 3D) and a higher ion leakage rate (Fig. 3E).

To examine the effect of *OsMYB102* on drought tolerance in

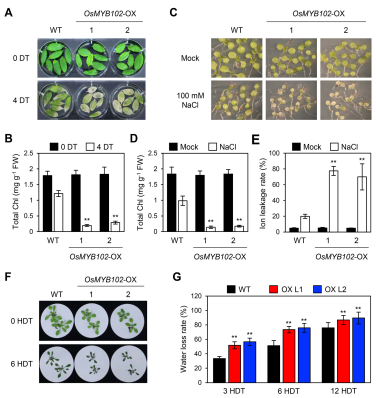

Fig. 3. The transgenic expression of *OsMYB102* increases susceptibility to high salinity and drought stresses in Arabidopsis. (A) Rosette leaves detached from four-week-old Col-0 (WT) and two independent *OsMYB102*-OX lines were transferred into 3 mM MES buffer (pH 5.8) containing 150 mM NaCl, and the changes of visible phenotypes (A) and total chlorophyll (Chl) contents (B) were examined before and after 4 d of treatment (DT). (C-E) The 5-d-old WT and two independent *OsMYB102*-OX lines grown on MS agar were transferred into MS agar containing 100 mM NaCl. The visible phenotypes (C), total Chl contents (D), and membrane ion leakage rates (E) were examined at 3 DT. (F, G) Four-week-old WT and two independent *OsMYB102*-OX lines (L1, L2) were placed on dry filter paper and dehydrated for indicated hours. The phenotypes were observed at 6 h of dehydration treatment (6 HDT) and the water loss was determined at 3, 6, and 12 HDT. Mean and SD values were obtained from more than five biological replicates (Student's *t*-test, *P < 0.05, **P < 0.01).

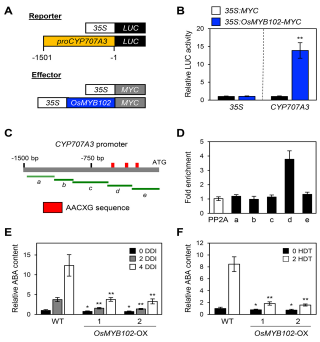

Fig. 4. The transgenic expression of *OsMYB102* inhibits ABA accumulation by upregulating *AtCYP707A3* transcription. (A) Reporter and effector constructs used in the transactivation assay. Each construct also contained the NOS terminator (not shown). (B) The activation of *AtCYP707A3* promoters (−1501 to −1 from the start codon) by the OsMYB102-Myc protein in a protoplast transient expression assay. The 35S promoter was used as the negative control. (C) The positions of the AACXG binding motif (red vertical lines) in the promoter of *AtCYP707A3*, and the promoter fragments used for the ChIP assay (green horizontal lines). (D) The *in planta* binding affinity of OsMYB102 to the promoter region of *AtCYP707A3*, examined using ChIP assays. The OsMYB102-Myc fusion protein was transiently expressed in protoplasts isolated from three-week-old Col-0 (WT) rosette leaves. The fold-enrichment of the promoter fragments was measured using an immunoprecipitation with an anti-Myc antibody. *PP2A* was used as the negative control. (E, F) Relative ABA contents in the leaves of the WT and two independent *OsMYB102*-OX lines during DIS (E) or dehydration (F). Mean and SD values were obtained from more than four biological samples. Asterisks indicate a significant difference from the WT or the negative control (Student's *t*-test, *P < 0.05, **P < 0.01).

Arabidopsis, the whole plants of WT and two independent *OsMYB102*-OX lines were transferred onto dry filter paper. After 6 h of the drying treatment (6 HDT), the *OsMYB102*-OX plants were almost completely wilted, while the WT plants seemed to have retained some freshness (Fig. 3F). Consistent with the visible phenotypes, the *OsMYB102*-OX plants lost significantly more of their relative fresh weights than the WT (Fig. 3G). These results demonstrate that the transgenic expression of *OsMYB102* enhances plant susceptibility to abiotic stresses, including salt and drought stresses, which is probably caused by OsMYB102-mediated modulation of a wide range of genes related to ABA accumulation and signaling.

OsMYB102 directly binds to the promoter of *AtCYP707A3* and induces its transcription in Arabidopsis

In rice, OsMYB102 inhibits ABA accumulation by directly inducing the expression of *OsCYP707A6*, which encodes an ABA catabolic enzyme (13). In this study, we found that *AtCYP707A3* and *AtCYP707A4*, the Arabidopsis homologs of *OsCYP707A6*, were also upregulated in the *OsMYB102*-OX plants (Fig. 2F). We previously showed that OsMYB102 has the capacity to bind to the AACXG consensus sequence, which is also known to be the binding motif of the closest OsMYB102 homolog in Arabidopsis, AtMYB44 (13, 20). We therefore examined the promoters of *AtCYP707A3* and *AtCYP707A4* (−1500 to −1 from the start codon). We found that the promoter of *AtCYP707A3* has four AACXG sequences (Fig. 4A), but the *AtCYP707A4* promoter does not contain this motif.

We used a protoplast transient expression assay to determine the activity of OsMYB102 on the induction of *AtCYP707A3* transcription. For the reporter construct, the promoter of *AtCYP707A3* (−1502 to −1 from the start codon) was fused with the luciferase (LUC) reporter, while *35S:OsMYB102-MYC* was used as the effector construct (Fig. 4B). We found that the LUC activity of protoplasts expressing *proAtCYP707A3:LUC* increased when they were co-transfected with the effector plasmid *35S:OsMYB102-MYC* (Fig. 4C). Next, with the Arabidopsis protoplasts expressing *OsMYB102-MYC*, we investigated the binding activity of OsMYB102 into the promoter region of *AtCYP707A3*. By a ChIP assay, we found that OsMYB102 bound to *AtCYP707A3* promoter region d, which contains an AACXG motif (Fig. 4D), indicating that OsMYB102 directly activates *AtCYP707A3* transcription in Arabidopsis.

To examine the effect of the activation of *AtCYP707A3* transcription by OsMYB102, we measured the ABA contents in the WT and two *OsMYB102*-OX lines during DIS and dehydration. The ABA levels in the *OsMYB102*-OX lines were lower than those in the WT even under normal growth conditions, although this difference was magnified during the DIS and dehydration treatments (Fig. 4E, F), indicating that overexpression of *OsMYB102* down-regulates ABA accumulation in Arabidopsis, at least in part by directly inducing the expression of *AtCYP707A3*.

DISCUSSION

In this study, we showed that transgenic Arabidopsis plants expressing *OsMYB102* have a strong delayed-senescence phenotype during DIS and were more susceptible to salinity and drought stresses (Figs. 2, 3). These phenotypes of *OsMYB102*-OX plants resembled those of Arabidopsis transgenics overexpressing *AtMYB44* which is the sequence homolog and probable functional homolog of *OsMYB102* (10, 21). In addition, a delayed senescence phenotype was also observed in the rice *osmyb102*-D mutant and rice *OsMYB102*-OX plants (13). The transgenic expression of *OsMYB102* inhibited the accumulation of ABA in Arabidopsis (Fig. 4), and ChIP and protoplast transient expression assays suggest that OsMYB102 directly activates the transcription of *AtCYP707A3* (Fig. 4) which is the homolog of *OsCYP707A6* (13). ABA promotes leaf senescence and also enhances tolerance to abiotic stresses (19, 22); therefore, probably the decrease of ABA accumulation by overexpression of *OsMYB102* led to delayed senescence phenotype and a decrease in plant tolerance to abiotic stresses such as salinity and drought (Figs. 2, 3).

In Arabidopsis, *OsMYB102*-OX plants also showed shorter petiole and semi-dwarf phenotypes under normal growth conditions (Fig. 1), similar to *AtMYB44*-OX (21). It was previously reported that Arabidopsis transgenics overexpressing *AtCYP707A3* and an ABA-deficient mutant *aba2-2* showed a semi-dwarf phenotype (23), similar to *OsMYB102*-OX (Fig. 1). Such a semi-dwarf phenotype is one of the typical phenotypes of ABA-deficient mutants, and probably it is caused by pleiotropic effects of ABA deficiencies, such as decrease of stomata closure rate when it is necessary. Collectively, these results strongly indicate that the physiological roles of OsMYB102 and its homologs are highly conserved, and that these genes may act as key regulators of ABA metabolism and signaling in various plant species.

We also identified some differences between Arabidopsis and rice plants expressing *OsMYB102*. We found that the Arabidopsis *OsMYB102*-OX plants were hypersensitive to high salinity and drought stresses (Fig. 3); however, this was not previously observed in rice *osmyb102-D* or *OsMYB102*-OX plants (13). So far, it is not clear why the overexpression of *OsMYB102* in rice does not alter its tolerance to abiotic stresses. One possibility is that, in rice, OsMYB102 has an important role in regulating other signaling pathways, diminishing the effect of this TF on abiotic stress tolerance. Further phenome and transcriptome analyses are necessary for elucidating the transcriptional regulatory networks mediated by OsMYB102.

MATERIALS AND METHODS

Plant materials and growth conditions

Arabidopsis thaliana Columbia-0 (Col-0) seeds were obtained from the Arabidopsis Biological Resource Center (ABRC). The Arabidopsis plants were grown on soil at 22-24°C in long-day conditions (16 h light/8 h dark) in a growth room equipped

with cool-white fluorescent lights (100 μmol m^{-2} s^{-1}). DIS was induced as previously described (24). Briefly, detached leaves were kept in 3 mM MES (pH 5.8) buffer in full darkness, or whole plants were transferred into full darkness.

Plasmid construction and transformation
The *OsMYB102* cDNA was amplified using RT-PCR with gene-specific primers (Supplementary Table S1) and sub-cloned into the pCR8/GW/TOPO plasmid, after which it was ligated into the pEarleyGate 203 gateway binary vector containing the 35S promoter (25). The recombinant plasmids were transformed into *Agrobacterium tumefaciens* strain GV3101. The *Agrobacterium*-mediated transformation into WT (Col-0) was performed using the floral-dip method (26). Transgenic T_0 seedlings were selected on the 0.5× MS phytoagar medium supplemented with 2 mM MES buffer (pH 5.8) and 12 mg l^{-1} phosphinothricin. The phosphinothricin-resistant seedlings were planted and reselected to obtain the final T_2 homozygous lines.

Reverse transcription-quantitative real-time PCR (RT-qPCR)
For the RT-qPCR analysis, the total RNA was isolated from Arabidopsis rosette leaves using the TRIzol reagent (Thermo Fisher Scientific). The first-strand cDNAs were prepared using 5 μg total RNA in a 25-μl reaction volume using M-MLV reverse transcriptase and oligo$(dT)_{15}$ primers (Promega), then diluted with water to 100 μl. The 20-μl qPCR mixture contained 1 μl cDNA, 10 μl 2× SYBR Green PCR Master Mix (Qiagen), and 0.25 mM each of the forward and reverse gene-specific primers (Supplementary Table S1). The qPCR analysis was carried out using a LightCycler 480 (Roche Diagnostics). To determine the relative expression, the mRNA abundance of each gene was normalized to that of *AtGAPDH* (At1g16300).

Chlorophyll (Chl) and carotenoid (Car) quantification
To measure total Chl and Car concentrations, the pigments were extracted from leaf tissues using 80% ice-cold acetone. The concentrations were determined using spectrophotometry, as described previously (27).

Measurement of ion leakage rates
Ion leakage was measured as described previously (28). Briefly, the membrane leakage was determined by measuring the loss of electrolytes (or ions) from the rosette leaves or seedlings. Ten leaves from each treatment were immersed in 6 ml of 0.4 M mannitol at room temperature and gently shaken for 3 h. The initial conductivity of the solution was then measured with a conductivity meter (CON6 Meter; LaMotte). After a 20-min incubation at 85°C, the total conductivity of the sample was determined. The rate of ion leakage was expressed as the percentage of initial conductivity divided by the total conductivity.

Stress treatments
The detached leaves and seedlings were exposed to a salt treatment as previously described (29). The dehydration treatment on dry filter paper was carried out following a previously described protocol (27).

Chromatin Immunoprecipitation assay
Plants expressing *OsMYC102-MYC* were grown in continuous white light for 7 d before being cross-linked for 20 min with 1% formaldehyde under vacuum. The chromatin complexes were isolated and sonicated as previously described (30), using an anti-MYC polyclonal antibody (Abcam) and Protein A agarose/salmon sperm DNA (Millipore) for the immunoprecipitation. After reverse cross-linking and protein digestion, the DNA was purified using a QIAquick PCR Purification Kit (Qiagen).

Co-transfection and transient expression in Arabidopsis protoplasts
To construct the reporter plasmids containing the *LUC* reporter gene under the control of *AtCYP707A3* promoter, the promoter fragment of *AtCYP707A3* (−1501 to −1 from the start codon) was cloned into the pGreenII-0579 vector, which combined the *LUC* reporter sequence with the gene of interest at its C-terminus. The plasmid containing *35S:OsMYC102-MYC* was previously generated (13). The reporter (4 μg) and effector plasmids (8 μg) were co-transfected into 5×10^4 Arabidopsis protoplasts by polyethylene glycol-mediated transfection (31). The transfected protoplasts were then suspended in protoplast culture medium (0.4 mM mannitol, 15 mM $MgCl_2$, and 4 mM MES, pH 5.8) and kept in the dark for 16 h. The LUC activity in each cell lysate was determined using the Luciferase Assay System Kit (Promega).

Quantification of ABA contents
To determine the ABA contents, three-week-old plants were dark-incubated or dehydrated for the indicated times, then collected and weighed. The leaves were ground in liquid nitrogen and homogenized with 80% methanol containing 1 mM butylated hydroxytoluene, then incubated overnight at 4°C to extract the endogenous ABA. The ABA contents were analyzed using the ABA ELISA Kit (MyBiosource).

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CONFLICTS OF INTEREST

The authors have no conflicting interests.

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