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Research

ORA47 (octadecanoid-responsive AP2/ERF-domain transcription factor 47) regulates jasmonic acid and abscisic acid biosynthesis and signaling through binding to a novel *cis*-element

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Summary

- *ORA47* (*octadecanoid-responsive AP2/ERF-domain transcription factor 47*) of *Arabidopsis thaliana* is an AP2/ERF domain transcription factor that regulates jasmonate (JA) biosynthesis and is induced by methyl JA treatment. The regulatory mechanism of *ORA47* remains unclear. ORA47 is shown to bind to the *cis*-element (NC/GT)CGNCCA, which is referred to as the O-box, in the promoter of *ABI2*. We proposed that ORA47 acts as a connection between *ABA INSENSITIVE1* (*ABI1*) and *ABI2* and mediates an ABI1–ORA47–ABI2 positive feedback loop.
- *PORA47:ORA47-GFP* transgenic plants were used in a chromatin immunoprecipitation (ChIP) assay to show that ORA47 participates in the biosynthesis and/or signaling pathways of nine phytohormones. Specifically, many abscisic acid (ABA) and JA biosynthesis and signaling genes were direct targets of ORA47 under stress conditions.
- The JA content of the *P35S:ORA47-GR* lines was highly induced under wounding and moderately induced under water stress relative to that of the wild-type plants. The wounding treatment moderately increased ABA accumulation in the transgenic lines, whereas the water stress treatment repressed the ABA content.
- ORA47 is proposed to play a role in the biosynthesis of JA and ABA and in regulating the biosynthesis and/or signaling of a suite of phytohormone genes when plants are subjected to wounding and water stress.

Introduction

Plants encounter many environmental stressors, such as drought, extreme temperature, wounding, hypoxia and salinity, and abscisic acid (ABA) accumulation is triggered by certain abiotic stresses, such as water deficits, wounding, extreme temperatures, and high salinity, as well as by biotic stresses. ABA also promotes resistance to salt, low temperatures, and drought stress (Finkelstein & Gibson, 2002) as well as pathogen attack (Asselbergh *et al.*, 2008; Ton *et al.*, 2009). The core machinery of the ABA signaling pathway includes type 2C protein phosphatases (PP2Cs) and type 2 sucrose nonfermenting-related kinases (SnRK2s) (Cutler *et al.*, 2010), which regulate ABA-dependent gene expression (Raghavendra *et al.*, 2010).

ABA INSENSITIVE1 (ABI1) and ABI2 are two important PP2Cs that function with the PYRABACTIN RESISTANCE 1-RELATED HOMOLOGS of *A. thaliana* (PYLs) as co-receptors, and act as negative regulators of ABA sensitivity (Raghavendra *et al.*, 2010). Although ABI1 and ABI2 are closely related, each has specific functions. For example, ABI2 physically interacts with the auxin signaling protein RHO-RELATED PROTEIN FROM PLANTS 11/*A. thaliana* RAC-LIKE 10 (ROP11/ARAC10), thereby enhancing its activity and decreasing ABA sensitivity (Yu *et al.*, 2012). ABI2 also negatively regulates GUARD CELL HYDROGEN PEROXIDE-RESISTANT 1 (GHR1), which can phosphorylate SLOW ANION CHANNEL-ASSOCIATED 1 (SLAC1) in stomatal closure (Hua *et al.*, 2012). The *abi2-1* mutation, but not the *abi1-1* mutation, disrupts ABA and methyl jasmonate (MeJA) signaling downstream of reactive oxygen species and nitric oxide production (Munemasa *et al.*, 2007).

Jasmonic acid (JA) is known to have important roles in mediating defense responses to biotic and abiotic stresses and in regulating normal growth and development (Wasternack & Hause, 2013). Perception of wounding or pathogen attack can trigger JA production. JA then activates several defense mechanisms. In addition, JAs are required for male fertility in *Arabidopsis thaliana* as well as fruit ripening and root growth (Wasternack & Hause, 2013).

As these examples illustrate, plant development and stress responses are orchestrated by hormone signaling pathways that may exhibit synergistic or antagonistic crosstalk to fine-tune plant

physiology (Grant & Jones, 2009; Depuydt & Hardtke, 2011). In plants, auxins, gibberellins (GAs), JA, ethylene, brassinosteroids (BRs), ABA, salicylic acid (SA), cytokinins (CKs), and strigolactone (SL) are essential for plant development, growth, and environmental responses (Grant & Jones, 2009; Wolters & Jürgens, 2009; Depuydt & Hardtke, 2011; Krouk *et al.*, 2011). Although a certain amount of the crosstalk in hormone signaling involves protein–protein interactions, such as with the DELLA protein (Sun, 2011), most of the mechanisms of hormone cross-regulation are not well understood. Transcriptional regulation is presumed to play an integral role in hormone crosstalk; however, whether this regulation involves the competing actions of different transcription factors or hormone-dependent effects on the activity of a few specific transcription factors has not been determined.

In this study, we investigated water stress- and ABA-induced transcription factors in the crosstalk among different signaling pathways. We screened seed pools of the *A. thaliana* TF ORF-EXpression (*At*TORF-EX) library (Weiste *et al.*, 2007) and found that plants that overexpressed *ORA47* (*octadecanoid-responsive AP2/ERF-domain transcription factor 47*; At1g74930) presented retarded growth and water stress sensitivity. *ORA47* encodes a member of the DEHYDRATION RESPONSE ELEMENT-BINDING PROTEIN A-5 (DREB A-5) subfamily of the AP2/ERF domain transcription factor family (Nakano *et al.*, 2006). Expression of *ORA47* was induced in roots under salt stress (Ma *et al.*, 2006) and strongly induced by MeJA and wounding (Walley *et al.*, 2007; Wang *et al.*, 2008; Wasternack & Hause, 2013; Rehrig *et al.*, 2014). *ORA47* regulates the expression of LIPOXYGENASE 3 (*LOX3*) by binding to its promoter and is involved in regulating JA biosynthesis (Pauwels *et al.*, 2008). Moreover, we present evidence that ORA47 binds to a new *cis*-element in the promoter of *ABI2* and acts as a signaling component between *ABI1* and *ABI2*. We propose that ORA47 plays an important role in the biosynthesis of JA and ABA and possibly ethylene, SA and strigolactone (SL). In addition, we suggest that ORA47 is also involved in regulating GA, BR, CK, and indole-3-acetic acid (IAA) signaling genes under both normal and stress conditions.

Materials and Methods

Plant material and growth conditions

The *Arabidopsis thaliana* (L.) Heynh ecotypes Columbia-0 (Col-0) and Landsberg erecta (Ler-0) were used in this study. *Arabidopsis thaliana* transformations performed with Col-0 included the *ORA47-RNAi*, *PORA47:GUS*, *P35S:ORA47-glucocorticoid receptor* (*GR*) and *PORA47:ORA47-GFP* transgenic lines. To generate the *ORA47-RNAi* transgenic plants, forward and reverse oligo primers of a gene-specific *ORA47* cDNA fragment (CCAAGACATTGTCAAGGGAGAAGAAG AATCGG GTTTAGTACCCGGATCCG) were synthesized and annealed using PCR and then cloned into pCR8 and the RNAi vector pB7GWIWG2(I) (VIB, Ghent, Belgium). The primers ORA47-5 and ORA47-3 were used for the full-length open reading frame (ORF) of *ORA47*, whereas ORA47-P-1 and ORA47-P-2 were used for a 1.4-kb promoter region of *ORA47*. The primers ORA47-P-1 and ORA47-3 were used for *PORA47:ORA47* cloning. The sequences of the primers are listed in Supporting Information Table S1. A cloned vector was introduced into the *Agrobacterium tumefaciens* strain GV3101, and wild-type (WT) *A. thaliana* plants were transformed by floral dipping. The seeds of these plants were sterilized with 0.5% bleach and 0.1% Triton X-100 and then plated on ½ Murashige & Skoog (MS) medium with 1% sucrose and 0.7% agar. To induce synchronous germination, the seeds were treated with vernalization in dark conditions for 3 d and then transferred to a walk-in growth chamber at 22°C under a 16 h : 8 h, light : dark photoperiod.

Water stress and wounding treatment

The water stress and wounding conditions were modified from a previous study (Kilian *et al.*, 2007). For the water stress treatment, the plants were grown on a ½ MS plate for 12–14 d and then air-dried using laminar flow with the cover removed for 15 min followed by recovery for 15 min before sample harvesting. In the wounding treatment, forceps were used to pinch the leaves of 2-wk-old plants, and then the plants were allowed to recover for 30 min.

Quantitative real-time PCR analysis

For details of the quantitative real-time PCR (qRT)-PCR analysis, see Methods S1.

Determination of proline, abscisic acid, 12-oxo-phytodienoic acid and jasmonic acid contents

For details of the determination of proline, ABA, 12-oxo-phytodienoic acid (OPDA) and JA contents, see Methods S2.

Stomatal aperture measurement

For the stomatal aperture measurements, epidermal peels were stripped from fully expanded leaves of 5-wk-old plants and floated in a solution of 30 mM KCl and 10 mM MES-KOH (pH 6.15) in Petri dishes. After incubation for 2.5 h under white light at 22°C to induce stomatal opening, different concentrations of ABA, 100 μM $CaCl_2$ or 1 mM SA (Khokon *et al.*, 2011; Mori *et al.*, 2006; Ramírez *et al.*, 2009) were added under light for another 2.5 h. The stomatal apertures were recorded with an Olympus BX51 system microscope and analyzed using DP-PSW software (Olympus, Shinjuku-ku, Tokyo, Japan). More than 130 stomata of the *P35S:ORA47 OVEREXPRESSED 3* (*OE3*) and WT plants each were measured using the ImageJ 1.36b software (Broken Symmetry Software; http://brokensymmetry.com).

Protoplast transient expression assay

The *A. thaliana* protoplast transient expression assay has been described previously (Yoo *et al.*, 2007). The effectors, reporters, and control vectors were obtained from Dr Masaru Ohme-Takagia (Gene Discovery Research Center, National Institute of

Advanced Industrial Science and Technology, Tsukuba, Japan) (Ohta *et al.*, 2001) and Dr Chiu-Ping Cheng (Institute of Plant Biology, National Taiwan University, Taipei, Taiwan). Full-length or partial *ORA47* ORF sequences were individually cloned into pK2GW7 (VIB) as effectors (see Fig. S2a later), and the same effector construction strategy was used for *ABI1*, *A. thaliana HOMEOBOX PROTEIN 6* (*AtHB6*), and *ORA47* (see Fig. 3b later). The reporters were constructed using the pKGWL7 vector (VIB). Primers ORA47-P-1 and ORA47-P-2 were used to clone a 1.4-kb region of the *ORA47* promoter (see Fig. 3b later). The AtHB6 binding site with a point mutation (PORA47-PM) was constructed based on a PCR strategy using the primers P-ORA47-PM-1.2 and P-ORA47-PM-2.2 (Table S1).

The reporter construct PABI2 (see Fig. 4a later) containing the 1015-bp *ABI2* promoter region between −1015 and −1 was cloned based on a PCR strategy using the primers P-ABI2-Fw and P-ABI2-Rv2, whereas the reporters PABI2-1, PABI2-2, and PABI2-3 were generated by digesting the *ABI2* promoter with the *Eco*RI and *Alu*I restriction enzymes at positions −679 and −404. The 1-kb *ABI2* promoter DNA was digested with *Apo*I at −438 and −179 to generate PABI2-4 and digested with *Msp*I at −912 and *Spe*I at −616 to generate PABI2-5. A 34-bp reporter was cloned with the primer 34-TATA and reporter-R, and PABI2△cis was cloned with P-ABI2-Fw, P-ABI2-Rv2, ABI2 Tcis-1, and ABI2 Tcis-2 (Table S1).

ORA47-GR system and microarray analysis

For details of the *ORA47-GR* microarray analysis, see Methods S3.

Gel mobility shift assay

The ORA47-(GST) Glutathione S-transferase recombination protein preparation and a gel mobility shift assay were performed as previously described (Urao *et al.*, 1993). A 588-bp ORF of *ORA47* was cloned into the pGEX-6P-1 vector at the *Eco*RI site and then introduced into the *Escherichia coli* strain DE3. The probe CABI2 (TTTTTTGTCGACCAATTTTGATTAA AGCTC) was end-labeled in the *ABI2* promoter between −432 and −402 using Digoxigenin (DIG) as the hot probe. An unlabeled probe was used for the competition assays. The DIG-labeled probes Cp, CABI1, CABI2, and M1–M13 were generated using PCR, and the sequences are listed in Fig. 4 (see later).

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) was performed according to Gendrel *et al.* (2005) using *c.* 2 g of tissue from the *PORA47:ORA47-GFP* transgenic lines and WT plants. Twelve-day-old plants grown on the ½ MS plate under normal conditions or treated with water stress or wounding were harvested to run the ChIP assay. In the water stress treatment, the plants were dried using laminar airflow for 15 min and then allowed to recover for 15 min, and they were then harvested for the ChIP assay. For the wounding treatment, forceps were used to pinch the leaves, and then the plants were allowed to recover for 30 min. The chromatin was sonicated to shear to *c.* 500 bp. After centrifugation (13 000 ***g***; 4°C), the chromatin in the supernatant was measured using a NanoDrop analyzer (Thermo Scientific, Waltham, MA, USA). The samples with equal amounts of chromatin were immunoprecipitated with 15 µl of GFP antibody (GFP-Trap®_M; ChromoTek, Hauppauge, NY, USA). The relative fold enrichment of the input controls (samples without GFP antibody immunoprecipitation) and bound DNA was evaluated using real-time PCR. KAPA SYBR Premix ExTaq was used for the real-time PCR (KAPA Biosystems, Woburn, MA, USA), which was performed with a Bio-Rad MyiQTM real-time PCR system (Bio-Rad, Hercules, CA, USA). The results of the real-time PCR were analyzed with iQ5 software (Bio-Rad). *ACTIN 2* and a noncoding (NC) sequence at position −1721 from the *ABI2* transcription start site were used as internal controls to normalize the results. Three individual biological samples were evaluated.

Results

Morphology of *P35S:ORA47* transgenic plants and *ORA47* expression patterns

Three individual plants presenting retarded growth were selected from the screened seed pools containing OE AP2/ERF transcription factors (Weiste *et al.*, 2007). The sequencing of OE plants identified three independent ORA47 lines: *P35S:ORA47 OE1*, *OE2*, and *OE3* (Fig. 1a). The *ORA47* mRNA expression level of the *P35S:ORA47* OE lines was also analyzed (Fig. 1b). T_3 and T_4 homozygotes of these lines were used for further characterization. The rosette diameter of 35-d-old WT plants was between 7 and 10 cm, whereas the rosette diameters of the *P35S:ORA47* plants were 2 and 4 cm. The bolting time of *P35S:ORA47* was *c.* 35 d, which was *c.* 7 d after the bolting time of the WT plants when grown under normal conditions. A water stress test was applied to the 3-wk-old plants, and the *P35S:ORA47* plants were more sensitive to water stress relative to the WT plants. A similar result was obtained in the water loss test, which was performed by drying 14-d-old rosettes without roots on the bench top and then weighing every 10 min to measure the rate of water loss (Fig. 1c).

Because the *P35S:ORA47* plants were sensitive to dehydration, we suspected that their endogenous proline level might be different from that of the WT plants under water stress. The *OE3* and WT plants were grown under normal soil conditions for 2 wk, and then water was withheld for 7 d. The proline level was significantly higher in the WT plants than in the *P35S:ORA47* plants after 7 d of water stress; however, differences were not observed when the plants were grown under normal conditions and at the early stage of water stress (Fig. 1d). We further investigated the seed germination sensitivity to ABA. At 1 µM ABA, the germination rate of the WT seeds dropped to 40%, whereas the *P35S:ORA47* seeds maintained a rate of 80–100% (Fig. 1e). The germination rates of the *P35S:ORA47* seeds treated with 2–3 µM ABA were also much higher than that of the WT seeds, indicating that *P35S:ORA47* seed germination is insensitive to ABA.

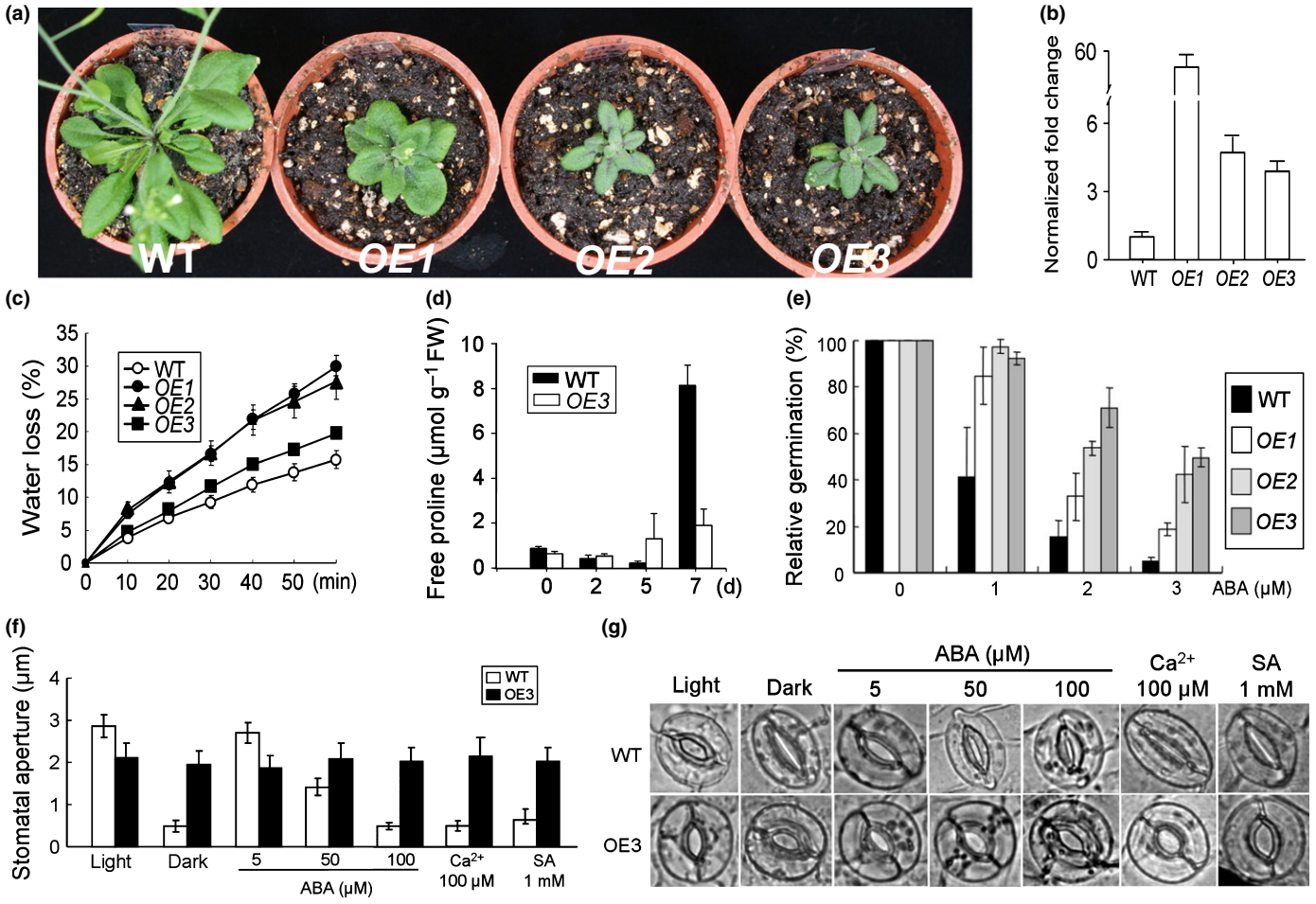


Fig. 1 Phenotype of the *ORA47* (*octadecanoid-responsive AP2/ERF-domain transcription factor 47*) overexpression (OE) lines and gene expression patterns of *ORA47*. (a) Vegetative phenotypes of the 35-d-old wild-type (WT) and *P35S:ORA47 OE1*, *OE2* and *OE3 Arabidopsis thaliana* plants. (b) *ORA47* mRNA expression level in the WT and *P35S:ORA47 OE1*, *OE2* and *OE3* plants. Values are presented as the mean ± SD ($n = 3$). (c) Water loss rates of the WT (open circles) and *P35S:ORA47 OE1* (closed circles), *OE2* (triangles), and *OE3* (squares) rosettes. Values are presented as the mean ± SD ($n = 5$). (d) Proline accumulation in the *P35S:ORA47 OE3* and WT plants under the drought treatment. The data represent the mean ± SD of three independent replications. (e) Seed germination rates of WT and *P35S:ORA47* with or without abscisic acid (ABA) treatment. One hundred seeds were used in each sample. Values are presented as the mean ± SD ($n = 4$). (f) Stomatal aperture was treated with light and dark conditions and ABA, Ca^{2+} and salicylic acid (SA). Values are presented as the mean ± SD ($n > 130$). The experiment was repeated three times with the same trend. (g) Micrographs representing changes in the WT and *P35S:ORA47 OE3* stomatal widths corresponding to the treatments in (f).

We also measured stomatal apertures using leaf epidermal strips treated with light, dark and ABA. These results indicated that *ORA47* overexpression reduced the sensitivity of stomatal aperture to dark and ABA (Fig. 1f,g). We further examined stomatal movements in response to applied Ca^{2+} or SA, also known to close the stomata (Mori *et al.*, 2006; Khokon *et al.*, 2011). These results also showed that the stomatal aperture of *ORA47OE* lines did not response to Ca^{2+} or SA either (Fig. 1f,g). Transient expression in protoplasts showed that *ORA47-GFP* was localized in the nucleus (Fig. S1), which is consistent with the function of ORA47 as a transcription factor.

ORA47 expression was induced by water stress, abscisic acid and wounding

To determine the *ORA47* expression patterns under stress, we dried 2-wk-old *A. thaliana* plants for 15 min, and then allowed them to recover for another 15 min. *ORA47* mRNA induction was detected in the first 15 min of water stress and reached a peak at 2 h (Fig. 2a). *ORA47* expression was also tested by spraying 10 μM ABA on the 2-wk-old WT plants. In response to ABA, *ORA47* induction was observed at 15 min and peaked at 30 min, and it subsequently declined dramatically (Fig. 2b).

Histochemical β-glucuronidase (GUS) staining was used to compare *ORA47* induction among the water stress, wounding and ABA treatments. A 1.4-kb promoter region of *ORA47* was used to prepare *PORA47:GUS* transgenic plants. WT and *PORA47:GUS* T_3 homozygous transgenic plants grown on ½ MS plates were used for GUS staining. GUS staining was detected in the vascular tissue at the cotyledon tips of the *PORA47:GUS* plants before the stress treatments (Fig. 2c2,c3). *ORA47* was highly expressed in response to water stress, wounding, and application of 10 μM ABA (Fig. 2c4–c12). However,

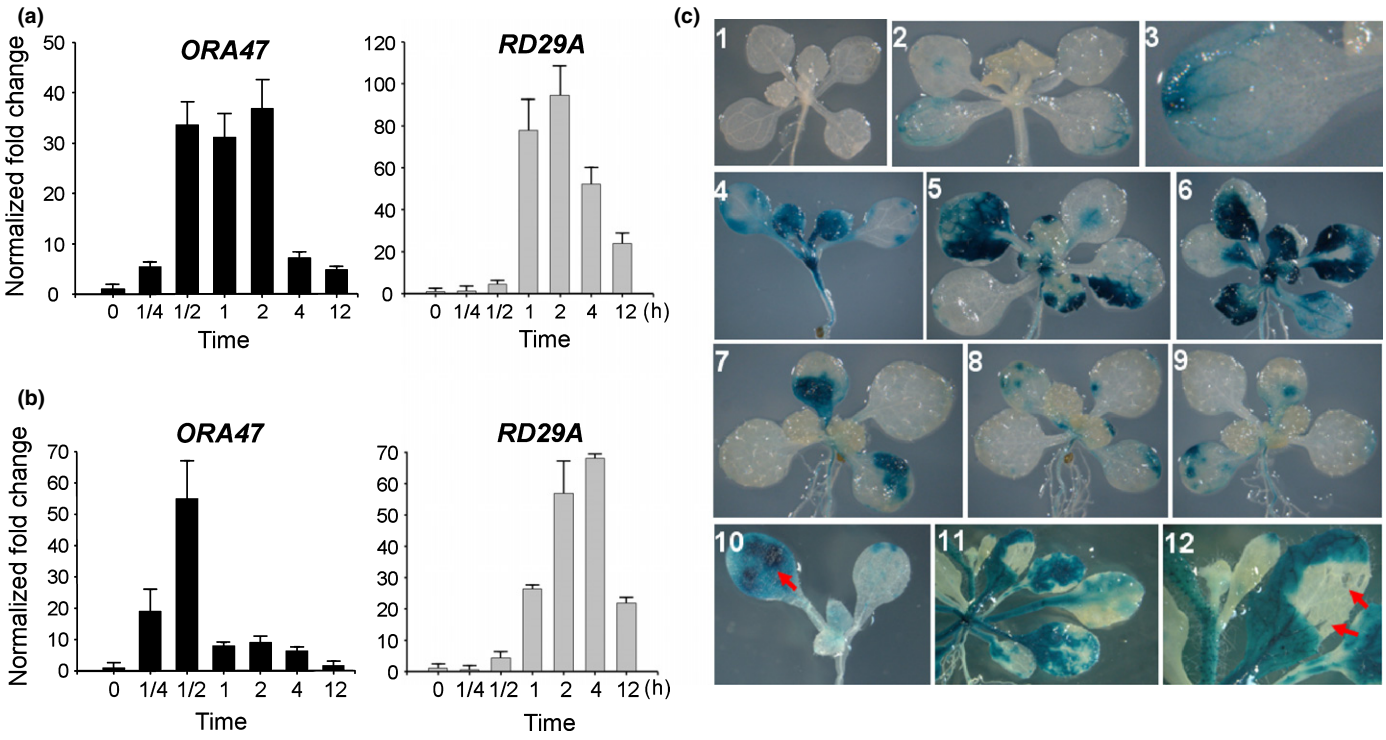


Fig. 2 *ORA47* (*octadecanoid-responsive AP2/ERF-domain transcription factor 47*) expression was induced by drought stress, abscisic acid (ABA) and wounding. (a) *ORA47* mRNA was induced by water stress. Two-week-old *Arabidopsis thaliana* plants were air-dried for 15 min, and the expression of *ORA47* was monitored for 12 h. *RESPONSIVE TO DESICCATION 29A* (*RD29A*) expression was included for comparison. Values are presented as the mean ± SD ($n = 3$). (b) *ORA47* mRNA was induced by ABA. Two-week-old wild-type (WT) plants were sprayed with 10 μM ABA, and the expression of *ORA47* was monitored for 12 h. *RD29A* expression was included for comparison. Values are presented as the mean ± SD ($n = 3$). (c) β-glucuronidase (GUS) staining of the *PORA47:GUS* transgenic plants treated with water stress, ABA or wounding. (1) GUS staining of a 2-wk-old WT plant. (2, 3) GUS staining of two independent transgenic lines of *PORA47:GUS* (T3 homozygote) under normal conditions. (4–6) *PORA47:GUS* transgenic plants showed strong GUS staining when treated with 15 min of water stress followed by a 3-h recovery period. (7–9) *PORA47:GUS* transgenic plants showed a GUS pattern when sprayed with 10 μM ABA followed by a 3-h recovery period. (10–12) *PORA47:GUS* transgenic plants showed strong GUS staining after they were pinched with forceps and allowed to recover for 3 h. (10) A 10-d-old plant was subjected to minor wounding at the cotyledon as indicated by a red arrow. (11, 12) A 3-wk-old plant was folded and severely pinched with forceps. The red arrow in (12) shows the forceps traces.

the ABA induction of GUS activity was lower than that observed in response to water stress or wounding.

AtHB6 and ABI1 cooperatively activate *ORA47* expression

Based on our observations that *ORA47* mRNA expression was induced by water stress and ABA treatment, we searched the *ORA47* promoter for ABA-related *cis*-elements using AthaMap (http://www.athamap.de/). We found that a homeodomain-leucine zipper (HDZip) class I protein binding site (CAATTATTG) (Johannesson *et al.*, 2001) was located at a position between −651 and −642 (Fig. 3a). Himmelbach *et al.* (2002) reported that the homeobox protein 6 (AtHB6), a HDZip class I protein, physically interacts with ABI1 and regulates ABA signaling, and they also reported that, when the binding site is modified, AtHB6 loses its binding ability. To elucidate the roles of ATHB6 and ABI1 in *ORA47* expression activation, we produced luciferase reporter constructs containing the 1.4-kb *ORA47* promoter (PORA47) and a promoter with a mutation in the putative AtHB6 binding site (PORA47-PM) (Fig. 3b). We also cloned *P35S:ORA47*, *P35S:AtHB6*, and *P35S:ABI1* as effectors (Fig. 3b). The greatest activation was achieved by the combination of AtHB6 and ABI1 (Fig. 3c), whereas only a medium level of activation was observed with AtHB6 or ABI1 alone. When the PORA47-PM reporter was introduced with AtHB6 and ABI1 into the protoplasts, activation was not observed (Fig. 3c). The results suggested that activation of *ORA47* is involved in the binding of AtHB6 to the *ORA47* promoter. Thus, *ORA47* may be a direct target of AtHB6 activation, and ABI1 may also be required for the increased activation of *ORA47* expression. These results also indicated that *ORA47* mRNA expression could not be induced by ORA47 itself (Fig. 3c).

ORA47 regulates *ABI2* mRNA expression and binds to a novel *cis*-element in the *ABI2* promoter

Because the *P35S:ORA47* plants presented a drought-sensitive and ABA-insensitive phenotype (Fig. 1), we hypothesized that the ABA negative regulator PP2C might have been activated in the *ORA47* OE lines. When we analyzed the mRNA expression levels of the ABA-related genes in the *P35S:ORA47* transgenic plants, we found that *ABI2* expression in the *ORA47 OE1* and *OE3* lines were five-fold and two-fold higher than that of the

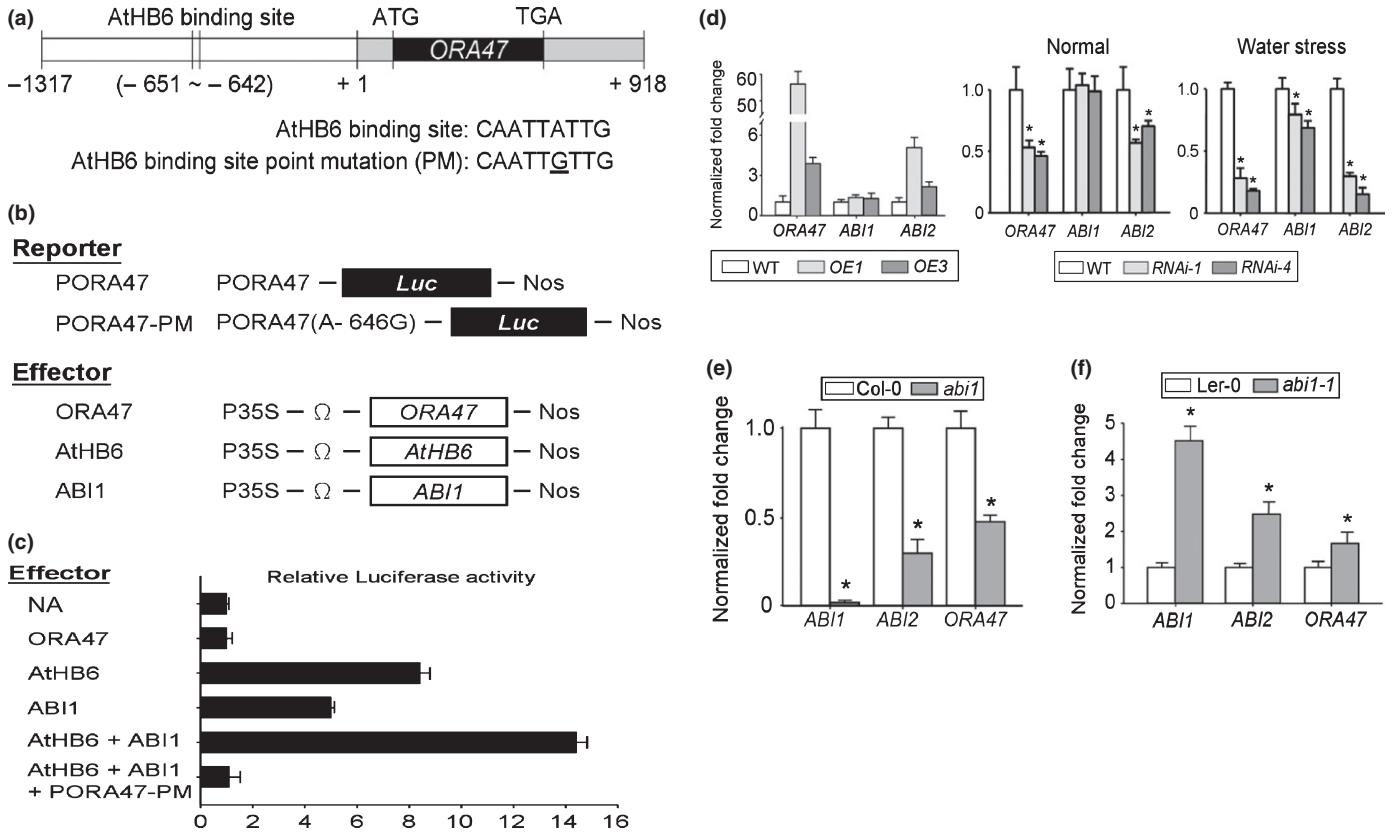


Fig. 3 *Arabidopsis thaliana HOMEOBOX PROTEIN 6* (*AtHB6*) *and ABA INSENSITIVE 1* (*ABI1*) cooperatively activate *ORA47* (*octadecanoid-responsive AP2/ERF-domain transcription factor 47*) expression, and *ABI2* expression was affected by ORA47. (a) Genomic structure of *ORA47*. The AtHB6 binding site is located at position −651 in the *ORA47* promoter. The white bar represents the promoter, the black bar represents the *ORA47* open reading frame (ORF), and the gray bars are 3′ and 5′ untranslated regions (UTRs). (b) Constructs of the *Arabidopsis thaliana* protoplast transient assay. The *ORA47* promoter or a mutated form drove the reporter (A-646G) (PORA47-PM). The effectors were full-length *ORA47*, *AtHB6*, or *ABI1* ORFs driven by the 35S promoter. (c) AtHB6 and ABI1 cooperatively activate *ORA47* expression. The relative luciferase activity of *PORA47:LUC* co-transformed with *P35S:ORA47* was indistinguishable from the background. Values are presented as the mean ± SD ($n = 5$). Three biological repeats showed the same trend. (d) *ORA47*, *ABI1* and *ABI2* mRNA expression in the wild-type (WT), *P35S:ORA47* overexpression (OE) lines and RNAi lines under normal conditions or after treatment with water stress. The plants were incubated in ½ Murashige & Skoog (MS) medium for 12 d under normal conditions. The same growth condition was applied in (e) and (f). (e) Lower *ABI1*, *ABI2*, and *ORA47* expression was observed in the *abi1* knockout mutant than in the WT plants. (f) Higher *ABI1*, *ABI2*, and *ORA47* expression was observed in the *abi1-1* dominant mutant than in the Ler-0 line. Values in (d)–(f) are represented as the mean ± SD. Three biological repeats were averaged. Statistically significant differences according to Student's *t*-test are indicated with asterisks (*, $P < 0.05$).

WT plants, respectively (Fig. 3d). We constructed *ORA47* RNAi lines for further study and found that the expression of *ABI2* mRNA in the *RNAi-1* and *RNAi-4* lines was *c.* 57% and 70% of the expression in the WT plants, respectively (Fig. 3d). Moreover, the morphology and drought resistance of the RNAi lines were not different from those of the WT plants. To confirm the regulatory relationship between *ABI*, *ORA47* and *ABI2* under stress conditions, 2-wk-old WT plants and *RNAi-1* and *RNAi-4* lines were subjected to water stress, and the expression of *ORA47* in the *RNAi-1* and *RNAi-4* lines was 28% and 18% of the expression in the WT plants, respectively, and the expression of *ABI2* in the *RNAi-1* and *RNAi-4* lines was 30% and 15% of the expression in the WT plants, respectively (Fig. 3d). However, the *ABI1* mRNA level in the *RNAi-1* and *RNAi-4* lines was approximately the same as that of the WT plants under normal conditions, whereas the level in the *RNAi-1* and *RNAi-4* lines was reduced to 79% and 69% under water stress, respectively. This result suggests that *ORA47* may play an important role in regulating *ABI2* gene expression under normal and water stress conditions but has less of an effect on *ABI1* gene expression. Accordingly, *ORA47* might act upstream of *ABI2*. ABI1 is a positive regulator of *ORA47* expression, and, to investigate whether *ABI1* acts upstream of *ORA47*, the *ABI1*, *ORA47* and *ABI2* mRNA levels were compared among 2-wk-old plants of the *ABI1* knockout line *abi1*, the dominant mutant line *abi1-1*, the Col-0 line, and the Ler-0 line. As shown in Fig. 3(e), *ABI2* expression in the *abi1* knockout mutant was reduced by 70% compared with that of the WT plants, and *ORA47* expression was reduced by half. However, the *ORA47* expression level was 1.7 times higher and the *ABI2* expression level was 2.5 times higher in the *abi1-1* mutant line relative to the Ler-0 line (Fig. 3f). Based on these results, which are shown in Fig. 3(e) and (f), we suggest that *ABI1* might act upstream of *ORA47* and *ABI2*.

To test the hypothesis that *ABI2* is a direct downstream target of ORA47 regulation, the promoter of *ABI2* was investigated. No known binding sequences of AP2/ERF domain transcription factors were found. The binding ability of ORA47 has not been investigated for the four known *cis*-elements of the AP2/ERF

domain transcription (Gong *et al.*, 2008). Subsequently, we found that ORA47 did not bind to a GCC box, which is one of the AP2/ERF binding *cis*-elements, and the transcriptional activation domain of ORA47 was located in the C-terminus between amino acids 139 and 195 (Fig. S2). This finding suggests that ORA47 might bind to an unknown *cis*-element in the *ABI2* promoter. To test this possibility, we generated a luciferase reporter controlled by 1015 bp of the *ABI2* promoter sequence (PABI2). A transient expression assay with *P35S:ORA47* as an effector showed that *ABI2* promoter activity was indeed induced by *P35S:ORA47* (Fig. 4a,b).

We further divided the *ABI2* 1015-bp promoter sequence into five overlapping fragments that included PABI2-1 (−1015/−679), PABI2-2 (−679/−405), PABI2-3 (−405/−1), PABI2-4 (−438/−179), and PABI2-5 (−912/−616), and each fragment was joined with a 140-bp 35S minimal promoter. Only PABI2-2 and PABI2-4 were activated by ORA47 (Fig. 4b; PABI2-2 and PABI2-4), and PABI2-2 overlapped with PABI2-4 by 34 bp (−438/−405). We then constructed a reporter containing only the 34-bp overlapping region fused to the 35S minimal promoter and found that this reporter was activated by ORA47 to a similar extent as the full-length promoter or PABI2-2 construct (Fig. 4b, 34 bp). We attempted to narrow down the 34 bp further but failed to obtain clear results. Accordingly, we propose that the ORA47-binding *cis*-element is located within this 34-bp promoter region (−438/−405) of *ABI2*.

ORA47-binding *cis*-element identification

To further elucidate the ORA47 binding sequence, we produced *P35S:ORA47-GR* transgenic lines. The ORA47-GR fusion protein binds to dexamethasone (DEX), a ligand of GR, and then translocates to the nucleus to regulate the mRNA expression of its target genes. To identify genes that are directly regulated by ORA47, the *P35S:ORA47-GR* transgenic line 4 was grown under normal conditions and treated with DEX and cycloheximide (CYC) to block protein synthesis to prevent secondary transcriptional regulation downstream of ORA47 regulation. The gene expression patterns were then analyzed using a microarray.

To exclude genes that were induced by DEX and CYC as well as genes from the transgenic plant background, analyses of two sets of microarrays were performed (Fig. S3). One microarray was composed of *P35S:ORA47-GR* transgenic plants treated with CYC for 1 h and then DEX for 2 h and *P35S:ORA47-GR* plants treated only with CYC as the control, and the other microarray was composed of *P35S:ORA47-GR* transgenic plants treated with CYC for 1 h and then DEX for 2 h and WT plants treated with DEX and CYC as the control. The data sets of the microarray analysis are available at the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/). The common up-regulated genes obtained from these two data sets were considered to represent the putative ORA47 direct downstream genes. The 1-kb promoter regions of the 100 most highly up-regulated genes (fold change > 4.1) were analyzed using the eTFBS program (http://biominer.csie.cyu.edu.tw/etfbs/) (Chen *et al.*, 2008). We found a conserved sequence, ACCGNC CAAT (N refers to A, T, G, C), in the promoter region of 49 of these 100 genes (Fig. 4c), and this putative *cis*-element was referred to as the O-box. The same ORA47-binding *cis*-element ((A/G)CCG(A/T)CC(A/T)) has also been found using protein-binding microarrays (Franco-Zorrilla *et al.*, 2014). A comparison of the 34-bp ORA47-binding region in the *ABI2* promoter (Fig. 4b) with the bioinformatic predictions (Fig. 4c) led us to hypothesize that the sequence GTCGACCAAT of the *ABI2* promoter (−426/−416) is the ORA47-binding *cis*-element responsible for ORA47 regulation of *ABI2*. The putative O-boxes from the 500 most up-regulated genes of the *P35S:ORA47-GR* microarray data set are listed in Table S2. We then created a reporter construct in which the putative ORA47-binding *cis*-element of the *ABI2* promoter (−426/−416) was deleted (PABI2△*cis*; Fig. 4a), and the results showed that the luciferase reporter activity was no longer induced by ORA47. Thus, the GTCGACCAAT sequence is responsible for ORA47 up-regulation of *ABI2* and is probably the site of ORA47 promoter binding.

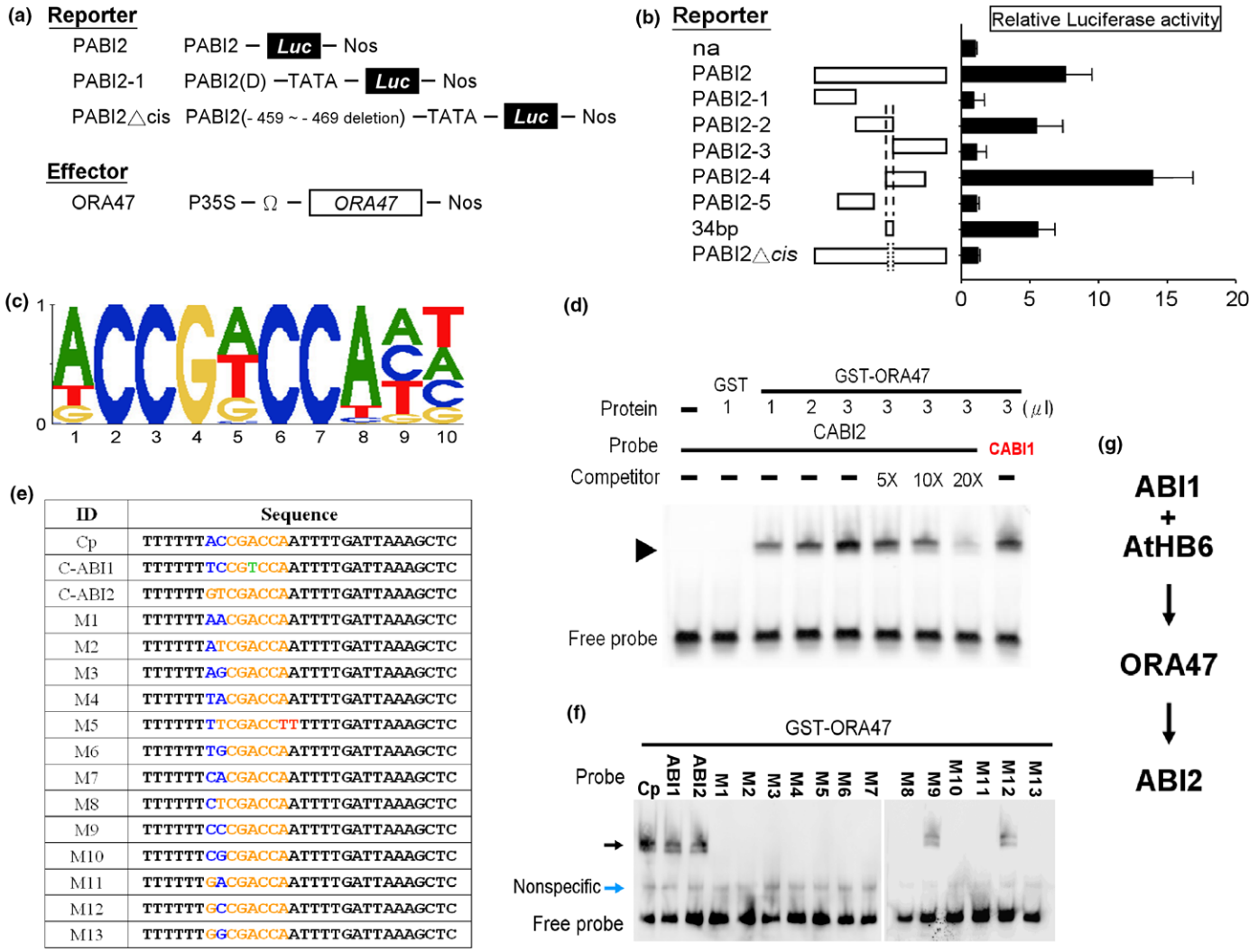
For the down-regulated genes in the microarray data set, enrichment of any specific *cis*-element was not observed using the eTFBS program. However, we manually searched the promoter region of the down-regulated genes and found that certain promoters contained the conserved O-box. A number of these promoters were selected for further experiments.

To demonstrate the direct binding of ORA47 to the putative O-box, we performed electromobility shift assays (EMSAs) using a recombinant GST-ORA47 fusion protein. As shown in Fig. 4(d), the 30-bp DIG-labeled probe CABI2 (probe sequence shown in Fig. 4e) specifically bound the DIG-labeled CABI2 probe (indicated by an arrow), although the addition of the unlabeled CABI2 probe reduced the extent of the binding. A probe containing the putative O-box in the ABI1 promoter (CABI1; TCCGTCCAAT) also showed ORA47 binding (Fig. 4d).

Because the O-box for ORA47 binding predicted by bioinformatics was slightly different from the O-box in the *ABI2* promoter, we designed another probe corresponding to the predicted O-box (ACCGACCAAT, labeled as Cp in Fig. 4e) and 13 other point-mutated probes (M1–M13) to test the importance of each base for ORA47 binding (Fig. 4e). ORA47 binding to the Cp probe was similar to the binding of CABI1 and CABI2. The probes M9 and M12 also showed relatively strong binding; however, other mutated probes did not bind ORA47 (Fig. 4f). Our findings support the model summarized in Fig. 4(g), whereby *ORA47* expression is regulated by the cooperative action of AtHB6 and ABI1, and ORA47 subsequently regulates the expression of *ABI2* under normal and water stress conditions. ORA47 might also regulate the expression of *ABI1*.

Based on the earlier analysis, the consensus ORA47 binding motif is (NC/GT) CGNCCA. Once this variation in the first two bases was taken into account, we observed that many of the genes up-regulated by ORA47 in Table S2 contained more than two O-boxes in their 1-kb promoter regions.

To identify the biological functions of the direct downstream genes regulated by ORA47, we scanned the 500 most up-regulated genes in the *P35S:ORA47-GR* microarray data set and



(e)

ID	Sequence
Cp	TTTTTTACCGACCAATTTTGATTAAAGCTC
C-ABI1	TTTTTTTCCGTCCAATTTTGATTAAAGCTC
C-ABI2	TTTTTTGTCGACCAATTTTGATTAAAGCTC
M1	TTTTTTAACGACCAATTTTGATTAAAGCTC
M2	TTTTTTATCGACCAATTTTGATTAAAGCTC
M3	TTTTTTAGCGACCAATTTTGATTAAAGCTC
M4	TTTTTTTACGACCAATTTTGATTAAAGCTC
M5	TTTTTTTTCGACCTTTTTGATTAAAGCTC
M6	TTTTTTTGCGACCAATTTTGATTAAAGCTC
M7	TTTTTTCACGACCAATTTTGATTAAAGCTC
M8	TTTTTTCTCGACCAATTTTGATTAAAGCTC
M9	TTTTTTCCCGACCAATTTTGATTAAAGCTC
M10	TTTTTTCGCGACCAATTTTGATTAAAGCTC
M11	TTTTTTGACGACCAATTTTGATTAAAGCTC
M12	TTTTTTGCCGACCAATTTTGATTAAAGCTC
M13	TTTTTTGGCGACCAATTTTGATTAAAGCTC

Fig. 4 Prediction of the ORA47 (octadecanoid-responsive AP2/ERF-domain transcription factor 47)-binding *cis*-element. (a) Constructs of the *Arabidopsis thaliana* protoplast transient assay for determining the ORA47 binding element. The reporter construct contained the *ABI2* promoter or the deletion variants of the *ABI2* promoter (PABI2(D)) that were driven by the 140-bp 35S minimal promoter and the TATA box. (b) Results of the protoplast transient assay. The scheme for the deletion variants of the *ABI2* promoter is shown on the left. Values are presented as the mean ± SD ($n = 5$). Three biological repeats showed the same trend. (c) Overrepresented motifs identified by the eTFBS program in the 1-kb promoter region of the up-regulated putative downstream genes obtained from the *P35S:ORA47-glucocorticoid receptor* (*GR*) microarray data set. The consensus sequence was referred to as the O-box *cis*-element. (d) GST-ORA47 fusion proteins bind specifically to the DIG-labeled probe CABI2 in the electromobility shift assay (EMSA). The shifted band indicated that GST-ORA47 binds to the CABI2 probe that was obtained from the promoter of *ABI2*. An unlabeled CABI2 probe was added in 5-, 10-, or 20-fold excess amounts in the competitive assay. CABI1 from the *ABI1* promoter was used for a positive comparison. Lane 1, probe only; lane 2, probe incubated with GST only. The arrow indicates the shifted banding. (e) List of Cp, CABI1, CABI2 and M1 to M13 point-mutated probes. (f) GST-ORA47 binds to Cp, CABI1, CABI2, M9 and M12 (arrow), and Cp shows stronger binding affinity than the other probes. (g) Working model summarizing the roles played by *ORA47*. ABI1 and AtHB6 cooperatively regulate the expression of ORA47, and ORA47 activates *ABI2* by binding to the O-box. *ABI1*, *ORA47*, and *ABI2* together modulate the negative regulation loop in the ABA signaling pathway. na, reporter only. *ABI2, ABA INSENSITIVE2; GST, Glutathione S-transferase;* DIG, Digoxigenin. *AtHB6, A. thaliana HOMEOBOX PROTEIN 6*.

identified 334 genes containing at least one putative O-box in their promoter region. A gene ontology analysis (http://bioinfo.-cau.edu.cn/agriGO/index.php) showed that ORA47 may be involved in developmental processes, stimulus responses, biological regulation, localization and transporter activity (Fig. S4) because these categories were overrepresented in the 344 putative ORA47-regulated genes. We also observed many phytohormone-related genes in the 334 up-regulated genes in Table S2. The Gene Ontology Consortium website (http://geneontology.org/) was used to identify genes categorized as phytohormones.

ORA47 binds to biosynthesis and/or signaling genes of multiple phytohormones

Table S2 shows that many phytohormone genes related to biosynthesis and signaling were up-regulated, suggesting that ORA47 might directly regulate the transcription of these genes. We selected 31 genes containing O-boxes for further validation of ORA47 binding (Fig. 5a; Table 1) and performed ChIP assays of the *PORA47:ORA47-GFP* transgenic plants subjected to several conditions. The *Actin 2* ORF and NC sequence, which are located

at position −1721 from the *ABI2* transcription start site (Fig. 5a), were used as internal negative controls in the ChIP assay.

Promoter binding of ORA47 was clearly dependent on both the specific gene under study and the environmental conditions to which the plants were exposed. For example, ORA47 bound to the O-box of the *ABI1* and *ABI2* promoters only under normal conditions and not under stress conditions. Similarly, the ABA biosynthesis genes *NINE-CIS-EPOXYCAROTENOID DIOXYGENASE 3* (*NCED3*) and *NCED9* and the ABA-responsive gene *RESPONSIVE TO DESICCATION 26* (*RD26*) were regulated by ORA47 under normal and wounding conditions but not under water stress conditions. ORA47 presented

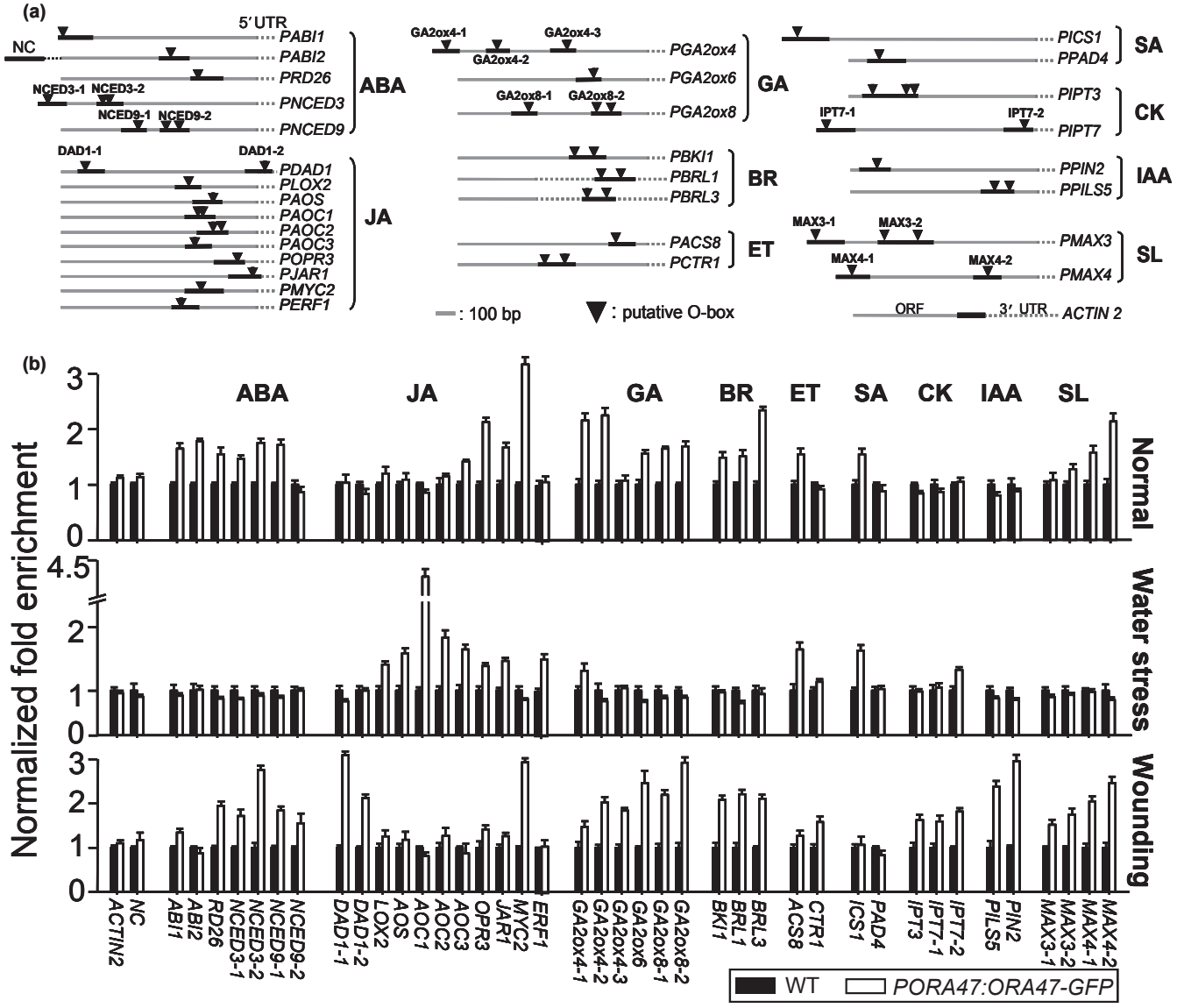


Fig. 5 ORA47 (octadecanoid-responsive AP2/ERF-domain transcription factor 47) of *Arabidopsis thaliana* binds to genes belonging to different phytohormones under normal, water stress and wounding conditions. (a) Putative O-boxes were found in the promoters of the jasmonic acid (JA), abscisic acid (ABA), ethylene (ET), brassinosteroid (BR), salicylic acid (SA), indole-3-acetic acid (IAA), cytokinin (CK), gibberellin (GA) and strigolactone (SL) biosynthesis or signaling genes. Arrows indicate a putative O-box. Short black lines indicate the region of the primer used for the chromatin immunoprecipitation (ChIP) assay. Primers specific for the *ACTIN 2* 3′ open reading frame (ORF) region and a noncoding (NC) sequence at position −1721 from the *ABI2* transcription starting site were used as internal controls. The scale indicates 100 bp. (b) Twelve-day-old wild-type (WT) (closed bars) and *PORA47:ORA47-GFP* (open bars) transgenic plants were treated with water stress or wounding before the ChIP assay. Results of the ChIP assay were grouped into categories of different phytohormones. Values are presented as the mean ± SD ($n = 3$). One of the three biological repeats that showed a similar trend is presented. Promoter of genes in (a): *PABI1, ABA INSENSITIVE 1 PABI2, ABA INSENSITIVE 2; PRD26, RESPONSIVE TO DESICCATION 26; PNCED3 and 9, NINE-CIS-EPOXYCAROTENOID DIOXYGENASE 3 and 9; PDAD1, DEFECTIVE ANTHER DEHISCENCE 1; PLOX2, LIPOXYGENASE 2; PAOS, ALLENE OXIDE SYNTHASE; PAOC1, 2 and 3, ALLENE OXIDE CYCLASE 1, 2 and 3; POPR3, OXOPHYTODIENOATE-REDUCTASE 3; PJAR1, JASMONATE RESISTANT 1; PMYC2, MYC2; PERF1, ETHYLENE RESPONSE FACTOR 1; PGA2ox4, 6 and 8, GIBBERELLIN 2-OXIDASE 4, 6 and 8; PBKI1, BRI1 KINASE INHIBITOR 1; PBRL1 and 3, BRI1-LIKE 1 and 3; PACS8: 1-AMINO-CYCLOPROPANE-1-CARBOXYLATE SYNTHASE 8; PCTR1, CONSTITUTIVE TRIPLE RESPONSE 1; PICS1, ISOCHORISMATE SYNTHASE 1; PPAD4, PHYTOALEXIN DEFICIENT 4; PIPT3 and 7, ISOPENTENYLTRANSFERASE 3 and 7; PPIN2, PIN-FORMED 2; PPILS5, PIN-LIKES 5; PMAX3 and 4, MORE AXILLARY BRANCHING 3 and 4.*

Table 1 O-box in the promoters of abscisic acid (ABA)-, jasmonic acid (JA)-, gibberellin (GA)-, brassinosteroid (BR)-, ethylene-, salicylic acid (SA)-, cytokinin (CK)-, indole-3-acetic acid (IAA)- and strigolactone (SL)-related genes

AGI	Gene name	Direction	Position	Sequence
ABA biosynthesis and signaling genes				
AT5G57050	ABI2	+	−459	GTCGACCAAT
AT4G26080	ABI1	+	−1141	TCCGTCCAAT
AT4G27410	RD26	+	−273	GTCGGCCAGT
AT3G14440	NCED3	−	−947	TCCGACCTTT
		−	−1370	TTCGACCACT
		+	−984	TGCGTCCGAA
AT1G78390	NCED9	+	−757	GTCGTCCGAC
		+	−521	TACGACCACA
		+	−320	ACCGTCCAAG
JA biosynthesis genes				
AT2G44810	DAD1	+	−976	GCCGTCCATT
		−	+27	TCCGGCCAAG
AT3G45140	LOX2	−	−397	ACCGGCCATT
AT5G42650	AOS	−	−182	ACCGGCCGAT
AT3G25760	AOC1	+	−296	ACCGGCCAAA
		+	−227	GCCGTCCATG
AT3G25770	AOC2	+	−211	ACCGGCCAAA
		+	−159	TCCGACCATG
AT3G25780	AOC3	+	−299	ACCGACCAAG
AT2G06050	OPR3	−	−90	ACCGACCATT
AT2G46370	JAR1	+	−5	TCCGTCCAAT
AT1G32640	MYC2	+	−236	TGCGACCAAA
AT3G23240	ERF1	−	−526	TACGACCTAA
GA metabolic process				
AT4G21200	GA2ox8	−	−694	ACCGTCCTTC
		−	−241	GCCGCCCATC
		−	−119	CACGTCCACC
AT1G02400	GA2ox6	+	−275	ACCGTCCCCA
AT1G47990	GA2ox4	+	−1215	CCCGACCAAA
		+	−985	ATCGGCCGGT
		−	−984	ACCGGCCGAT
		−	−409	TACGACCACT
BR biosynthesis and signaling genes				
AT5G42750	BKI1	+	−403	TCCGGCCATC
		+	−260	TCCGTCCGTC
AT1G55610	BRL1	+	+273	TCCGACCAGC
		+	+425	ATCGACCTTT
AT3G13380	BRL3	+	+281	TCCGACCATC
		−	+181	TACGACCACT
Ethylene biosynthesis and signaling genes				
AT4G37770	ACS8	−	−152	TCCGTCCACA
AT5G03730	CTR1	−	−552	AACGTCCACA
		−	−706	TTCGGCCTGC
SA biosynthesis genes				
AT1G74710	ICS1	+	−1481	CGCGTCCAAC
AT3G52430	PAD4	−	−902	GTCGTCCAAA
CK biosynthesis genes				
AT3G63110	IPT3	−	−677	ACCGTCCATT
		−	−713	GTCGACCTGC
		−	−963	CACGTCCATG
AT3G23630	IPT7	+	−1233	TGCGTCCGAA
		−	−83	TACGACCTTA
IAA biosynthesis genes				
AT5G57090	PIN2	+	−942	AACGCCCAAT
AT2G17500	PILS5	+	−270	CTCGTCCTAA
		+	−211	TTCGTCCTTA
SL biosynthesis genes				
AT2G44990	MAX3	+	−1367	ATCGACCAAT
		+	−921	GGCGCCCTTT
		−	−727	ATCGACCCAC
		−	−925	GGCGCCCATA
AT4G32810	MAX4	+	−1090	CTCGCCCAAA
		−	−322	GGCGACCAAA

ABI1, ABA INSENSITIVE 1; ABI2, ABA INSENSITIVE 2; RD26, RESPONSIVE TO DESICCATION 26; NCED3 and 9, NINE-CIS-EPOXYCAROTENOID DIOXYGENASE 3 and 9; DAD1, DEFECTIVE ANTHER DEHISCENCE 1; LOX2, LIPOXYGENASE 2; AOS, ALLENE OXIDE SYNTHASE; AOC1, 2 and 3, ALLENE OXIDE CYCLASE 1, 2 and 3; OPR3, OXOPHYTODIENOATE-REDUCTASE 3; JAR1, JASMONATE RESISTANT 1; ERF1, ETHYLENE RESPONSE FACTOR 1; GA2ox4, 6 and 8, GIBBERELLIN 2-OXIDASE 4, 6 and 8; BKI1, BRI1 KINASE INHIBITOR 1; BRL1 and 3, BRI1-LIKE 1 and 3; ACS8: 1-AMINO-CYCLOPROPANE-1-CARBOXYLATE SYNTHASE 8; CTR1, CONSTITUTIVE TRIPLE RESPONSE 1; ICS1, ISOCHORISMATE SYNTHASE 1; PAD4, PHYTOALEXIN DEFICIENT 4; IPT3 and 7, ISOPENTENYLTRANSFERASE 3 and 7; PIN2, PIN-FORMED 2; PILS5, PIN-LIKES 5; MAX3 and 4, MORE AXILLARY BRANCHING 3 and 4.

different binding patterns with the O-box in the promoters of JA biosynthesis genes, including *DEFECTIVE ANTHER DEHISCENCE 1* (*DAD1*), *LIPOXYGENASE 2* (*LOX2*), *ALLENE OXIDE SYNTHASE* (*AOS*), *ALLENE OXIDE CYCLASE 1, 2 and 3* (*AOC1*), *AOC2*, *AOC3*, *OXOPHYTODIENOATE-REDUCTASE 3* (*OPR3*), *JASMONATE RESISTANT 1* (*JAR1*), and signaling genes, including *MYC2* and *ETHYLENE RESPONSE FACTOR 1* (*ERF1*), under the three conditions. Furthermore, we discovered that O-boxes occur in the promoter regions of biosynthesis and signaling genes of additional phytohormones, including GA, BR, ethylene, SA, CK, IAA and SL (Fig. 5b; Table 1). For example, ORA47 bound to the O-boxes of genes related to GA deactivation, including *GIBBERELLIN 2-OXIDASE 4* (*GA2ox4*), *GA2ox6* and *GA2ox8* (Schomburg *et al.*, 2003; Wang *et al.*, 2004; Jasinski *et al.*, 2005), the BR signaling inhibitor *BRI1 KINASE INHIBITOR 1 (BKI1)*, and the *BRI1-LIKE 1 (BRL1)* and *BRL3* receptors (Caño-Delgado *et al.*, 2004) under normal and wounding conditions but not under water stress conditions.

The ethylene biosynthesis gene *1-AMINO-CYCLOPROPANE-1-CARBOXYLATE SYNTHASE 8* (*ACS8*), the signaling gene *CONSTITUTIVE TRIPLE RESPONSE 1* (*CTR1*), the SA biosynthesis gene *ISOCHORISMATE SYNTHASE 1* (*ICS1*), and the signaling gene *PHYTOALEXIN DEFICIENT 4* (*PAD4*) also contain the O-box. ORA47 might regulate *ACS8* and *ICS1* under normal and water stress conditions, whereas ORA47 appeared only to bind to the *CTR1* O-box under wounding conditions. However, *PAD4* was not controlled by ORA47 under any of the tested conditions (Fig. 5b). O-boxes were also found in the promoters of CK biosynthesis genes (*ISOPENTENYLTRANSFERASE 3* and *7* (*ITP3* and

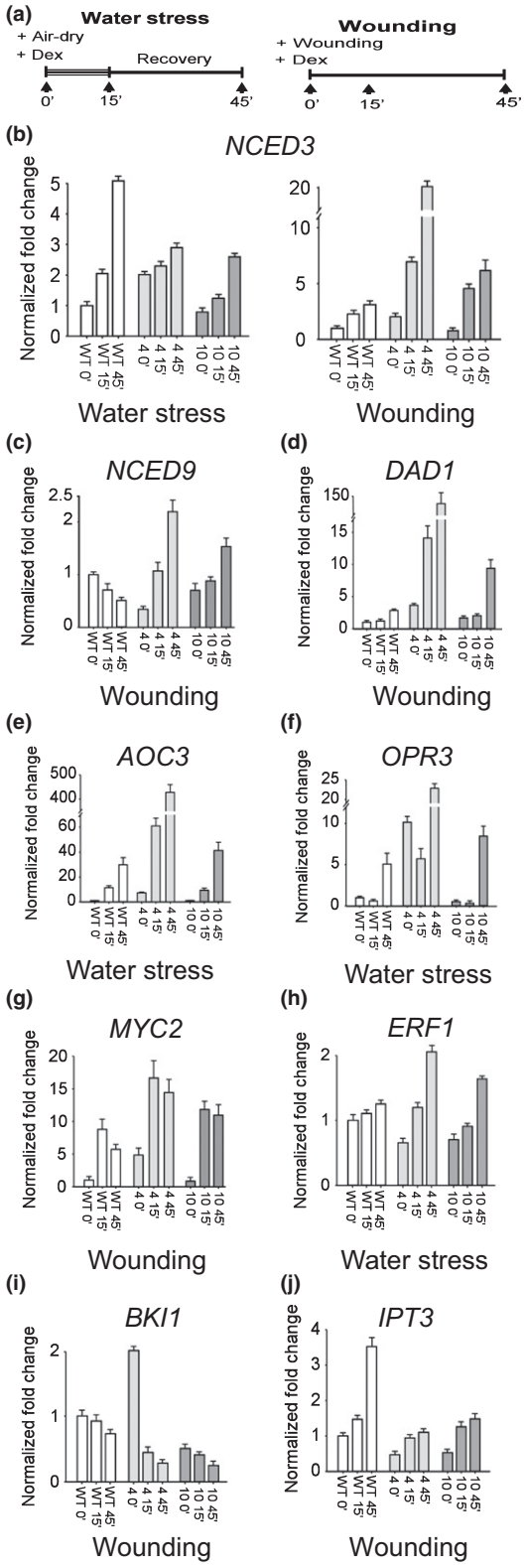


Fig. 6 ORA47 (octadecanoid-responsive AP2/ERF-domain transcription factor 47) of *Arabidopsis thaliana* acts as either a transcriptional activator or a repressor of the abscisic acid (ABA) and jasmonic acid (JA)-related downstream genes. (a) Scheme to show the effects of water stress and wounding treatment on the 12-d-old wild-type (WT) plants and *P35S:ORA47-glucocorticoid receptor* (*GR*) transgenic lines 4 and 10. Plants grown on plates were treated with 20 μM dexamethasone (DEX) and then subjected to either 15 min of dehydration followed by recovery for another 30 min or pinching with forceps. Samples were harvested at 0, 15 and 45 min. (b–j) Gene expression patterns of the WT and *P35S:ORA47-GR* transgenic lines 4 (4) and 10 (10) treated with DEX and water stress or DEX and wounding. Tested genes included (b) *NCED3*, (c) *NCED9*, (d) *DAD1*, (e) *AOC3*, (f) *OPR3*, (g) *MYC2*, (h) *ERF1*, (i) *BKI1* and (j) *IPT3*. To determine whether a gene was repressed or activated, we estimated the slope of three expression values at 0, 15 and 45 min for the WT and *P35S:ORA47-GR* transgenic lines. Gene expression values are presented as the mean ± SD ($n = 4$). Three biological repeats showed the same trend. *NCED3 and 9, NINE-CIS-EPOXYCAROTENOID DIOXYGENASE 3 and 9; DAD1, DEFECTIVE ANTHER DEHISCENCE 1; AOC3, ALLENE OXIDE CYCLASE 3; OPR3, OXOPHYTODIENOATE-REDUCTASE 3; ERF1, ETHYLENE RESPONSE FACTOR 1; BKI1, BRI1 KINASE INHIBITOR 1; IPT3, ISOPENTENYLTRANSFERASE 3*.

IPT7)), SL biosynthesis genes (*MORE AXILLARY BRANCHING 3 and 4* (*MAX3* and *MAX4*)), and IAA transporter genes (*PIN-LIKES 5* (*PILS5*) and *PIN-FORMED 2* (*PIN2*)). ORA47 was bound to the O-box of CK and IAA genes only under wounding conditions. Similar to ABA-, GA- and BR-related genes, the O-boxes in the promoter regions of the *MAX3* and *MAX4* genes were bound by ORA47 under both normal and wounding conditions. ORA47 was found to bind to the typical O-box as well as to the 'O-box-like' sequence. For example, ORA47 bound to the sequence TGCGACCAAA in the promoter of *MYC2* under normal and wounding conditions, AACGCCCAAT in the promoter of *PIN2* under wounding conditions, and TACGACCTAA in the promoter of *ERF1* under water stress conditions (Fig. 5; Table 1). Based on these results, we concluded that CGNCC is the core sequence of the O-box.

ORA47 might act as a transcriptional activator and repressor

In the ChIP assay discussed in the previous section, it was generally unclear how the pattern of ORA47 promoter associations in different conditions correlated with expression changes, either activation or repression. In the *P35S:ORA47-GR* microarray data set, we noticed that the up-regulated genes contained the O-box in their promoters and certain down-regulated genes also contained the O-box. This finding suggested that ORA47 could also mediate transcriptional suppression. To test the hypothesis that ORA47 acts as an activator and a repressor, 12-d-old WT plants and *P35S:ORA47-GR* transgenic lines 4 and 10 were treated with 20 μM DEX and then subjected to water stress or wounding (Fig. 6a), and the mRNA levels of nine selected genes that showed binding to the O-box in the ChIP assay were measured at 0, 15 and 45 min (Fig. 6b–j). To determine whether a gene is repressed or activated, we estimated the slope of the regression curve generated from the three expression values at 0, 15 and 45 min for the WT plants and *P35S:ORA47-GR* transgenic lines (Table S3). If a specific gene in the transgenic line presented a higher or lower slope than that of the WT, the gene was considered to be activated or repressed, respectively. Additionally, the gene expression patterns of the WT and *P35S:*

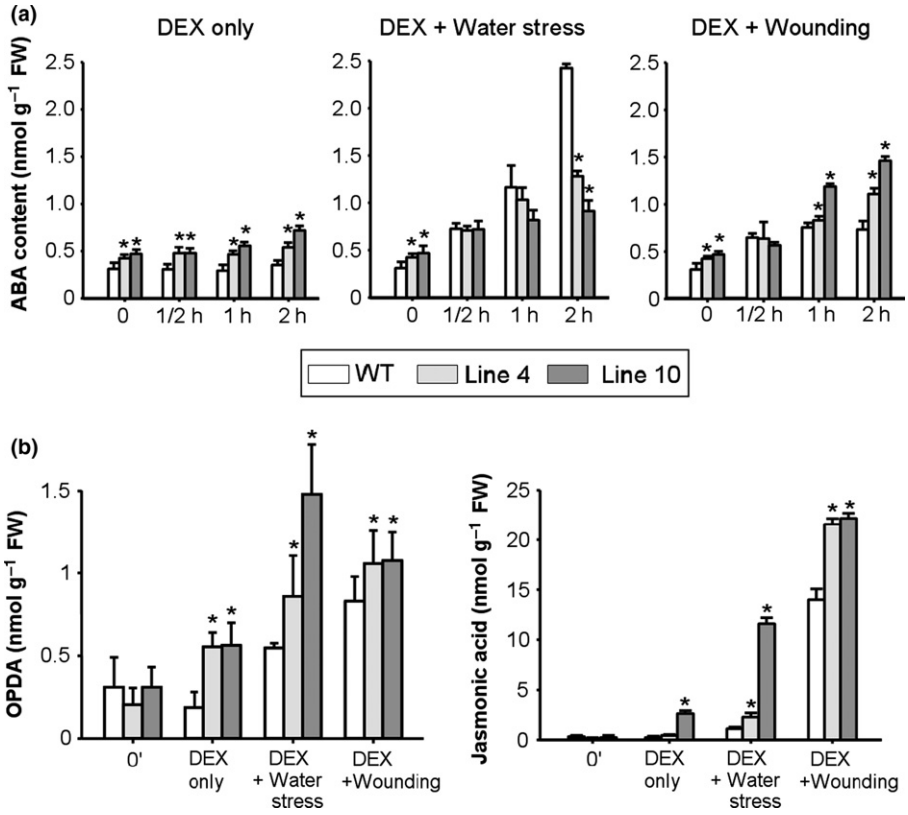


Fig. 7 *ORA47* (*octadecanoid-responsive AP2/ERF-domain transcription factor 47*) of *Arabidopsis thaliana* regulates abscisic acid (ABA), 12-oxo-phytodienoic acid (OPDA) and jasmonic acid (JA) contents under normal, water stress and wounding conditions. (a) Twelve-day-old wild-type (WT) plants (white bars) and *P35S:ORA47-glucocorticoid receptor* (*GR*) transgenic lines 4 (dark gray bars) and 10 (black bars) treated with normal (dexamethasone (DEX) only), water stress (DEX + water stress) and wounding (DEX + wounding) conditions for 0, ½, 1 and 2 h. ABA content in the transgenic lines was increased under the normal and wounding treatments but decreased under the water stress treatment relative to that of the WT plants. Asterisks indicate values that were significantly different from the WT at each time-point according to Student's *t*-test (*, $P < 0.05$). Error bars represent the SD of three biological replicates. (b) Twelve-day-old WT plants and *P35S:ORA47-GR* transgenic lines 4 and 10 were treated with DEX only, water stress (DEX + water stress) or wounding (DEX + wounding) for 1 h, and then the JA content was analyzed with LC-MS. JA and OPDA contents in lines 4 and 10 were higher after treatment with DEX in all three conditions compared with those of the WT plants. Asterisks indicate values that were significantly different from the WT in each experiment (*, $P < 0.05$).

ORA47-GR plants treated with DEX only and water stress or wounding without DEX were also analyzed (Fig. S5). This background information was useful for predicting whether ORA47 is an activator or repressor (for a further explanation, see the legend to Fig. S5). Accordingly, the genes *NCED3*, *NCED9*, *DAD1*, *IPT3* and *MYC2* under wounding and *AOC3*, *OPR3* and *ERF1* under water stress were found to be activated by *ORA47* overexpression, whereas the gene *BKI1* was repressed under wounding. *NCED3* did not present promoter binding under water stress but showed repressed gene expression, suggesting that *ORA47* overexpression might act as a repressor of *NCED3* expression under water stress. We propose that ORA47 might act as a transcriptional activator or repressor depending on the gene and the conditions.

ORA47 regulates JA and ABA accumulation under stress conditions

In the previous section, we showed that ORA47 activates ABA (*NCED3* and *NCED9*; Fig. 6b,c) and JA biosynthesis and signaling genes (*DAD1* and *MYC2*; Fig. 6d,g) in the *ORA47-GR* transgenic lines under wounding conditions. Moreover, under water stress, certain JA biosynthesis and signaling genes were activated (*AOC3* and *ERF1*; Fig. 6e,h), although the ABA biosynthesis gene *NCED3* was not (Figs 5b, 6b). Therefore, we hypothesized that JA content would be increased under both stresses, while the ABA content would only be increased under wounding in the *P35S:ORA47-GR* transgenic lines. To observe the physiological effect of *ORA47* expression under stress, it is essential to measure the concentrations of JA and ABA in the WT and *ORA47-GR* lines. Twelve-day-old WT plants and *P35S:ORA47-GR* transgenic lines 4 and 10 were treated with water stress or wounding, and the ABA and JA concentrations were analyzed (Fig. 7).

We observed that the ABA contents in lines 4 and 10 were higher than that in the WT plants under normal conditions (Fig. 7a), and the ABA content in lines 4 and 10 was slightly but significantly increased under the DEX treatment compared with that of the WT plants (Student's *t*-test). Water stress in the WT plants increased the ABA content, which showed an eight-fold increase at 2 h compared with that recorded at baseline, whereas only 3.7- and 2.5-fold increases were observed in lines 4 and 10,

respectively. Wounding for 2 h slightly but significantly increased the ABA accumulation in lines 4 and 10 compared with that in the WT plants (Student's *t*-test). Subsequently, we observed that the JA content was induced in transgenic lines after the DEX treatment under normal conditions (Fig. 7b). Wounding for 1 h greatly increased the JA accumulation in lines 4 and 10 compared with that in the WT plants (Student's *t*-test). Water stress also increased the JA content but to a lesser extent relative to that after wounding. These results are consistent with the results of the ChIP assay (Fig. 5) and the gene expression assay of the *P35S:ORA47-GR* transgenic lines subjected to the stress treatments (Fig. 6). Fig. 7(b) also shows that OPDA, an intermediate of JA biosynthesis, slightly increased under stress treatment for 1 h but still maintained low accumulation levels. The significance of the differences between the mean values for the DEX treatment, genotypes and stress treatments were assessed using a two-way ANOVA (Table S4). The ANOVA results were consistent with those of Student's *t*-test (shown in Fig. 7), thus indicating that *ORA47* overexpression indeed had a significant effect on ABA, OPDA and JA accumulation when the plant was under stress conditions.

Discussion

Plant stress responses are regulated by a complex network of cross-communicating signaling pathways. In the present study, we demonstrated that ORA47 binds to a novel *cis*-element present in a number of downstream genes involved in hormone biosynthesis and signaling as well as other processes. Intriguingly, ORA47 may also regulate nine phytohormones at the same time under normal and stress conditions and thus may be involved in the creation of suitable cell environments for plant survival.

The ABA co-receptors ABI1 and ABI2 bind to the PYRABACTIN RESISTANCE (PYR)/REGULATORY COMPONENTS OF ABA RECEPTORS (RCAR)–ABA complex and activate ABA signaling and ABA responses (Raghavendra *et al.*, 2010). Here, we propose that *ORA47* links *ABI1* and *ABI2* and mediates an ABI1–ORA47–ABI2 positive feedback loop. These data may explain the observation in which overexpressed AtHB6 is ABA insensitive (Himmelbach *et al.*, 2002). We predict that the generation of ABI2 is primarily mediated by ORA47 under normal and water stress conditions in *A. thaliana* (Figs 3d, 4g).

Global gene expression profiling has identified a number of *CORONATINE INSENSITIVE 1* (*COI1*)-dependent JA-regulated genes, including *ORA47* in *A. thaliana* (Devoto *et al.*, 2005; Jung *et al.*, 2007). The phenomenon of self-activation of JA biosynthesis genes by MeJA has been previously reported (Pauwels *et al.*, 2008), and we propose that the underlying mechanism may involve ORA47. In this study, we provide evidence that ORA47 may serve as a master regulator of JA biosynthesis and signaling and demonstrate that ORA47 is one of the early responsive transcription factors (Pauwels *et al.*, 2008), thus forming a positive feedback loop to promote JA signaling under stress. Consistent with the results of the promoter binding and gene expression assays, the JA content in the *P35S:ORA47-GR* transgenic lines was greatly increased under wounding. However, the JA content in the *P35S:ORA47-GR* transgenic lines after water stress was only moderately increased. In conclusion, we suggest that ORA47 plays an important role in JA biosynthesis/signaling upon wounding and is less important under water stress. JA plays a key role in controlling the resource allocation between the competing processes of growth and defense (Ullmann-Zeunert *et al.*, 2013), and JA treatment rapidly arrests leaf growth, which is consistent with the repression of genes involved in cell division and expansion (Pauwels *et al.*, 2008; Attaran *et al.*, 2014) and explains the growth arrest phenotype of the *ORA47* OE lines.

To our knowledge, the participation of ORA47 in ABA biosynthesis is a novel function. In the present study, ORA47 was found to bind to and activate ABA biosynthesis genes, including *NCED3* and *NCED9*, and the signaling gene *RD26* through the O-box in the genes' promoter regions. Consequently, ORA47 was found to act as a regulator of ABA biosynthesis and signaling. Consistent with the results of the promoter binding and gene expression assays, the ABA content in the *P35S:ORA47-GR* transgenic lines was moderately increased after wounding but not after water stress. Therefore, ORA47 plays a role in ABA biosynthesis/signaling under wounding but not under water stress.

We suggest that ORA47 might have a role in the hormonal interplay between signaling pathways because ORA47 binds to the promoters of various hormone-related genes. This mechanism may explain a number of findings from previous reports that observed crosstalk between hormones but were unable to identify the underlying mechanism. For example, JAs are involved in the regulation of auxin transport through the distribution of the auxin exporter PIN-FORMED 2 (PIN2) (Sun *et al.*, 2011). Our ChIP assay suggested that ORA47 is probably involved in this process (Fig. 5; Table 1). JA antagonizes GA biosynthesis in the stems of *Nicotiana attenuate* (Heinrich *et al.*, 2013), and the antagonism between JA and GA is probably related to the ORA47-induced increase of JA biosynthesis and GA deactivation through the activated expression of three *GA2ox* genes. JA signaling may have a positive effect on ABA signaling by inducing genes such as *PYRABACTIN RESISTANCE 1-LIKE 4* (*PYL4*) (Lackman *et al.*, 2011). Consistent with this activity, ORA47 induces the expression of *PYL5*, *PYL7*, and *PYL13,* which also contain the O-box (Table S2). JA and ethylene act synergistically and are mutually dependent in plant defense responses (Lorenzo *et al.*, 2003; Zhu *et al.*, 2011). Consistent with this activity, ORA47 promotes the expression of a number of JA biosynthesis genes and the *ACS8* gene under wounding and water stress conditions. Therefore, ORA47 probably functions as a master switch that regulates the biosynthesis and/or signaling of genes related to phytohormones under stress.

Certain transcription factors have dual functions. In *A. thaliana*, ABI4 is an ABA signaling responsive transcriptional activator that is active during seed maturation and seed germination and binds to the known coupling element 1 (CE1) *cis*-element (CAC(C/A)G). A role of ABI4 in repressing genes

activated by G-box (CACGTG) binding transcription factors has been proposed where ABI4 binding to the CE1 element blocks the binding of other transcription factors to the G-box (Wind *et al.*, 2013). Franco-Zorrilla *et al.* (2014) performed protein microarray analyses and showed that ORA47 also binds to the GCC box. However, a GCC or DRE box was not observed in the eTFBS program predictions. Using a ChIP assay under different conditions, we observed that ORA47 bound to an O-box-like *cis*-element. Interestingly, the O-box ((NC/GT)CGNCCA) has a similar sequence to the DRE (ACCGAC) box and GCC (GCCGCC) box, indicating that ORA47 might repress gene expression through competition for the binding site with other AP2 domain transcription factors. Although the regulatory mechanism is still unknown, we suggest that ORA47 has a dual effect on downstream genes under various conditions.

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Author contributions

H-Y.C. and T.P.L designed the research. H-Y.C. performed most of the experiments and analyzed the data. E-J.H. and M-C.C. performed some experiments. C-Y.C. and S-Y.H. contributed new computational tools and analyzed data. H-Y.C. and T-P.L wrote the manuscript.

References

Asselbergh B, Achuo AE, Hofte M, Van Gijsegem F. 2008. Abscisic acid deficiency leads to rapid activation of tomato defence responses upon infection with *Erwinia chrysanthemi*. *Molecular Plant Pathology* **9**: 11–24.

Attaran E, Major IT, Cruz JA, Rosa BA, Koo AJK, Chen J, Kramer DM, He SY, Howe GA. 2014. Temporal dynamics of growth and photosynthesis suppression in response to jasmonate signaling. *Plant Physiology* **165**: 1302–1314.

Caño-Delgado A, Yin Y, Yu C, Vafeados D, Mora-Garcia S, Cheng JC, Nam KH, Li J, Chory J. 2004. BRL1 and BRL3 are novel brassinosteroid receptors that function in vascular differentiation in Arabidopsis. *Development* **131**: 5341–5351.

Chen CY, Tsai HK, Hsu CM, May Chen MJ, Hung HG, Huang GTW, Li WH. 2008. Discovering gapped binding sites of yeast transcription factors. *Proceedings of the National Academy of Sciences, USA* **105**: 2527–2532.

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.

Depuydt S, Hardtke CS. 2011. Hormone signalling crosstalk in plant growth regulation. *Current Biology* **21**: 365–373.

Devoto A, Ellis C, Magusin A, Chang HS, Chilcott C, Zhu T, Turner JG. 2005. Expression profiling reveals COI1 to be a key regulator of genes involved in wound- and methyl jasmonate-induced secondary metabolism, defence, and hormone interactions. *Plant Molecular Biology* **58**: 497–513.

Finkelstein RR, Gibson SI. 2002. ABA and sugar interactions regulating development: cross-talk or voices in a crowd? *Current Opinion in Plant Biology* **5**: 26–32.

Franco-Zorrilla JM, López-Vidrieroa I, Carrascob JL, Godoya M, Verab P, Solano R. 2014. DNA-binding specificities of plant transcription factors and their potential to define target genes. *Proceedings of the National Academy of Sciences, USA* **111**: 2367–2372.

Gendrel AV, Lippman Z, Martienssen R, Colot V. 2005. Profiling histone modification patterns in plants using genomic tiling microarrays. *Nature Methods* **2**: 213–218.

Gong W, He K, Covington M, Dinesh-Kumar SP, Snyder M, Harmer SL, Zhu YX, Deng XW. 2008. The Development of protein microarrays and their applications in DNA–protein and protein–protein interaction analyses of *Arabidopsis* transcription factors. *Molecular Plant* **1**: 27–41.

Grant MR, Jones JD. 2009. Hormone (dis)harmony moulds plant health and disease. *Science* **324**: 750–752.

Heinrich M, Hettenhausen C, Lange T, Wunsche H, Fang J, Baldwin IT, Wu J. 2013. High levels of jasmonic acid antagonize the biosynthesis of gibberellins and inhibit the growth of *Nicotiana attenuate* stems. *Plant Journal* **73**: 591–606.

Himmelbach A, Hoffmann T, Leube M, Hohener B, Grill E. 2002. Homeodomain protein ATHB6 is a target of the protein phosphatase ABI1 and regulates hormone responses in *Arabidopsis*. *EMBO Journal* **21**: 3029–3038.

Hua D, Wang C, He J, Liao H, Duan Y, Zhu J, Guo Y, Chen Z, Gong Z. 2012. A plasma membrane receptor kinase, GHR1, mediates abscisic acid- and hydrogen peroxide-regulated stomatal movement in *Arabidopsis*. *Plant Cell* **24**: 2546–2561.

Jasinski S, Piazza P, Craft J, Hay A, Woolley L, Rieu I, Phillips A, Hedden P, Tsiantis M. 2005. KNOX action in *Arabidopsis* is mediated by coordinate regulation of cytokinin and gibberellin activities. *Current Biology* **15**: 1560–1565.

Johannesson H, Wang Y, Engström P. 2001. DNA-binding and dimerization preferences of *Arabidopsis* homeodomain-leucine zipper transcription factors *in vitro*. *Plant Molecular Biology* **45**: 63–73.

Jung C, Lyou SH, Yeu S, Kim MA, Rhee S, Kim M, Lee JS, Choi YD, Cheong JJ. 2007. Microarray-based screening of jasmonate-responsive genes in *Arabidopsis thaliana*. *Plant Cell Reports* **26**: 1053–1063.

Khokon AR, Okuma E, Hossain MA, Munemasa S, Uraji M, Nakamura Y, Mori IC, Murata Y. 2011. Involvement of extracellular oxidative burst in salicylic acid-induced stomatal closure in Arabidopsis. *Plant, Cell and Environment* **34**: 434–443.

Kilian J, Whitehead D, Horak J, Wanke D, Weinl S, Batistic O, D'Angelo C, Bornberg-Bauer E, Kudla J, Harter K. 2007. The AtGenExpress global stress expression data set: protocols, evaluation and model data analysis of UV-B light, drought and cold stress responses. *Plant Journal* **50**: 347–363.

Krouk G, Ruffel S, Gutiérrez RA, Gojon A, Crawford NM, Coruzzi GM, Lacombe B. 2011. A framework integrating plant growth with hormones and nutrients. *Trends in Plant Science* **16**: 178–182.

Lackman P, Gonzalez-Guzman M, Tilleman S, Carqueijeiro I, Perez AC, Moses T, Seo M, Kanno Y, Hakkinen ST, Van Montagu MC *et al.* 2011. Jasmonate signaling involves the abscisic acid receptor PYL4 to regulate metabolic reprogramming in *Arabidopsis* and tobacco. *Proceedings of the National Academy of Sciences, USA* **108**: 5891–5896.

Lorenzo O, Piqueras R, Sánchez-Serrano JJ, Solano R. 2003. ETHYLENE RESPONSE FACTOR1 integrates signals from ethylene and jasmonate pathways in plant defense. *Plant Cell* **15**: 165–178.

Ma S, Gong Q, Bohnert HJ. 2006. Dissecting salt stress pathways. *Journal of Experimental Botany* **57**: 1097–1107.

Mori IC, Murata Y, Yang Y, Munemasa S, Wang YF, Andreoli S, Tiriac H, Alonso JM, Harper JF, Ecker JR *et al.* 2006. CDPKs CPK6 and CPK3 function in ABA regulation of guard cell S-type anion- and Ca^{2+}-permeable channels and stomatal closure. *PLoS Biology* **4**: 1749–1762.

Munemasa S, Oda K, Watanabe-Sugimoto M, Nakamura Y, Shimoishi Y, Murata Y. 2007. The coronatine-insensitive 1 mutation reveals the hormonal signaling interaction between abscisic acid and methyl jasmonate in Arabidopsis guard cells. Specific impairment of ion channel activation and second messenger production. *Plant Physiology* **143**: 1398–1407.

Nakano T, Suzuki K, Fujimura T, Shinshi H. 2006. Genome-wide analysis of the ERF gene family in Arabidopsis and rice. *Plant Physiology* **140**: 411–432.

Ohta M, Matsui K, Hiratsu K, Shinshi H, Ohme-Takagi M. 2001. Repression domains of class II ERF transcriptional repressors share an essential motif for active repression. *Plant Cell* **13**: 1959–1968.

Pauwels L, Morreel K, De Witte E, Lammertyn F, Van Montagu M, Boerjan W, Inzé D, Goossens A. 2008. Mapping methyl jasmonate-mediated transcriptional reprogramming of metabolism and cell cycle progression in cultured *Arabidopsis* cells. *Proceedings of the National Academy of Sciences, USA* **105**: 1380–1385.

Raghavendra AS, Gonugunta VK, Christmann A, Grill E. 2010. ABA perception and signalling. *Trends in Plant Science* **15**: 395–401.

Ramírez V, Coego A, López A, Agorio A, Flors V, Vera P. 2009. Drought tolerance in Arabidopsis is controlled by the OCP3 disease resistance regulator. *Plant Journal* **58**: 578–591.

Rehrig EM, Appel HM, Jones AD, Schultz JC. 2014. Roles for jasmonate- and ethylene-induced transcription factors in the ability of *Arabidopsis* to responds differentially to damage caused by two insect herbivores. *Frontiers in Plant Science* **5**: 407.

Schomburg FM, Bizzell CM, Lee DJ, Zeevaart JA, Amasino RM. 2003. Overexpression of a novel class of gibberellin 2-oxidases decreases gibberellin levels and creates dwarf plants. *Plant Cell* **15**: 151–163.

Sun J, Chen Q, Qi L, Jiang H, Li S, Xu Y, Liu F, Zhou W, Pan J, Li X *et al.* 2011. Jasmonate modulates endocytosis and plasma membrane accumulation of the Arabidopsis PIN2 protein. *New Phytologist* **191**: 360–375.

Sun TP. 2011. The molecular mechanism and evolution of the GA-GID1-DELLA signaling module in plants. *Current Biology* **21**: 338–345.

Ton J, Flors V, Mauch-Mani B. 2009. The multifaceted role of ABA in disease resistance. *Trends in Plant Science* **14**: 310–317.

Ullmann-Zeunert L, Stanton MA, Wielsch N, Bartram S, Hummert C, Svatoš A, Baldwin IT, Groten K. 2013. Quantification of growth defense trade-offs in a common currency: nitrogen required for phenolamide biosynthesis is not derived from ribulose-1,5-bisphosphate carboxylase/oxygenase turnover. *Plant Journal* **75**: 417–429.

Urao T, Yamaguchi-Shinozaki K, Urao S, Shinozaki K. 1993. An *Arabidopsis* myb homolog is induced by dehydration stress and its gene product binds to the conserved MYB recognition sequence. *Plant Cell* **5**: 1529–1539.

Walley JW, Coughlan S, Hudson ME, Covington MF, Kaspi R, Banu G, Harmer SL, Dehesh K. 2007. Mechanical stress induces biotic and abiotic stress responses via a novel cis-element. *PLoS Genetics* **3**: 1800–1812.

Wang H, Caruso LV, Downie AB, Perry SE. 2004. The embryo MADS domain protein AGAMOUS-Like 15 directly regulates expression of a gene encoding an enzyme involved in gibberellin metabolism. *Plant Cell* **16**: 1206–1219.

Wang Z, Cao G, Wang X, Miao J, Liu X, Chen Z, Qu LJ, Gu H. 2008. Identification and characterization of COI1-dependent transcription factor genes involved in JA-mediated response to wounding in *Arabidopsis* plants. *Plant Cell Reports* **27**: 125–135.

Wasternack C, Hause B. 2013. Jasmonates: biosynthesis, perception, signal transduction and action in plant stress response, growth and development. An update to the 2007 review in Annals of Botany. *Annals of Botany* **111**: 1021–1058.

Weiste C, Iven T, Fischer U, Oñate-Sánchez L, Dröge-Laser W. 2007. *In planta* ORFeome analysis by large-scale over-expression of GATEWAY®-compatible cDNA clones: screening of ERF transcription factors involved in abiotic stress defense. *Plant Journal* **52**: 382–390.

Wind JJ, Peviani A, Snel B, Hanson J, Smeekens SC. 2013. ABI4: versatile activator and repressor. *Trends in Plant Science* **18**: 125–132.

Wolters H, Jürgens G. 2009. Survival of the flexible: hormonal growth control and adaptation in plant development. *Nature Reviews Genetics* **10**: 305–317.

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

Yu F, Qian L, Nibau C, Duan Q, Kita D, Levasseur K, Li X, Lu C, Li H, Hou C *et al.* 2012. FERONIA receptor kinase pathway suppresses abscisic acid signaling in *Arabidopsis* by activating ABI2 phosphatase. *Proceedings of the National Academy of Sciences, USA* **109**: 14693–14698.

Zhu Z, An F, Feng Y, Li P, Xue L, A M, Jiang Z, Kim JM, To TK, Li W *et al.* 2011. Derepression of ethylene-stabilized transcription factors (EIN3/EIL1) mediates jasmonate and ethylene signaling synergy in *Arabidopsis*. *Proceedings of the National Academy of Sciences, USA* **108**: 12539–12544.

Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 The ORA47 protein is localized to the nucleus.

Fig. S2 Protoplast transient assay performed to map the activation domain of ORA47.

Fig. S3 Manipulation of the *ORA47-GR* transgenic plant using DEX and CYC to activate *ORA47-GR* for the microarray study.

Fig. S4 Functional categorization of genes directly bound by ORA47 using GO term enrichment.

Fig. S5 Gene expression pattern of ORA47 downstream genes under the DEX treatment, water stress and wounding.

Table S1 Primers used for the real-time PCR assay, ChIP assay, and plasmid construction

Table S2 List of 767 putative *cis*-elements from the first 500 up-regulated genes of the *ORA47-GR* microarray data set

Table S3 Slopes of the gene expression values in Fig. 6

Table S4 ANOVA results for the effects of ORA47 on the regulation of ABA, OPDA and JA content in plants (genotype) treated with DEX, water stress and wounding

Method S1 Quantitative real-time PCR analysis.

Method S2 Determination of proline, ABA, OPDA and JA content.

Method S3 ORA47-GR system and microarray analysis.

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